Martian superoxide and peroxide O_2 release (OR) assay: A new technology for terrestrial and planetary applications

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Running title: Metal superoxide/peroxide O_2 release assay

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Abstract

This study presents an assay for the detection and quantification of soil metal superoxides and peroxides in regolith and soil. The O_2 release (OR) assay is based on the enzymatic conversion of the hydrolysis products of metal oxides to O_2, and their quantification by an O_2 electrode based on the stoichiometry of the involved reactions: The intermediate product $O_2^{\bullet-}$ from the hydrolysis of metal superoxides is converted by cytochrome *c* to O_2, and also by superoxide dismutase (SOD) to ½ mol O_2 and ½ mol H_2O_2, which is then converted by catalase (CAT) to ½ mol O_2. The product H_2O_2 from the hydrolysis of metal peroxides and hydroperoxides is converted to ½ mol O_2 by CAT. The assay-method was validated in a sealed sample chamber using a liquid-phase Clark-type O_2 electrode with known concentrations of $O_2^{\bullet-}$ and H_2O_2, and with commercial metal superoxide and peroxide mixed with Mars analogue Mojave and Atacama Desert soils. Carbonates and perchlorates, both present on Mars, do not interfere with the assay. The assay lower limit of

detection, using luminescence quenching/optical sensing O_2-electrodes, is 1 nmol O_2 cm^{-3} or better. The activity of the assay enzymes SOD and cytochrome *c* was unaffected up to 6 Gy exposure by gamma-radiation, while CAT retained 100% and 40% of its activity at 3 and 6 Gy, respectively, demonstrating the suitability of these enzymes for planetary missions, e.g., in Mars or Europa.

Introduction

In 1976, the Viking Lander performed biological experiments designed to detect extant life in Martian soil. In the Viking Gas Exchange (GEx) experiment, up to ~770 nmol O_2 (g) was produced from soil samples (1 cm^3) upon humidification or wetting. The persistence of O_2 (g) release from samples that were heated to 145ºC for 3 hrs, and then cooled, prior to wetting or humidification ruled out any possible biological contribution to the GEx results **(Klein, 1978; Oyama *et al.*, 1977)**. In the Viking Labeled Release (LR) experiment, up to ~30 nmol ^{14}C labeled gas, presumed to be CO_2 was released after soil samples (0.5 cm^3) were wetted with an aqueous solution containing ^{14}C-labeled organics **(Klein *et al.*, 1976; Levin and Straat, 1976)**. The release of ^{14}C-labeled gas in the LR experiment was eliminated by heating the sample to 160ºC for 3 hrs, and then cooled, prior to the addition of the labeled aqueous organics. These results lead to the conclusion that the Martian surface material contains more than one type of reactive oxidant **(Klein, 1978)**. Metal superoxides were among the earliest proposed explanations for the thermally stable agent responsible for O_2 (g) release in the GEx experiment. In the case to the LR experiment, peroxide was among the earliest explanations proposed for the thermally liable agent responsible for $^{14}CO_2$ release. In addition to the possible presence of metal superoxides, it has been proposed that superoxide radical ($O_2^{\bullet-}$) is generated on the Martian dust and regolith surfaces by UV-induced mechanism **(Yen *et al.*, 2000)**. Such mechanism for $O_2^{\bullet-}$ photo-generation has been also shown with Mars analogue Mojave and Atacama soils **(Georgiou *et al.*, 2015)**.

More recently, high levels of soil perchlorate was measured at the Phoenix landing site **(Boynton *et al.*, 2009; Hecht *et al.*, 2009)** and its presence at the Viking and MSL landings has

been inferred based on thermal analyses **(Glavin *et al.*, 2013; Leshin *et al.*, 2013; Navarro-Gonzalez *et al.*, 2003)**. While the stability of perchlorate under the conditions of the GEx and LR preclude it as a direct explanation for these experiments, it has been suggested that perchlorate radiolysis products reproduce the major aspects of both experiments **(Quinn *et al.*, 2013)**. The form of the trapped O_2, in particular, derived from perchlorate radiolysis, was not identified and it has been suggested of some fraction may exist as superoxide or peroxide **(Prince and Johnson, 1965; Quinn *et al.*, 2013)**.

Given the poorly understood nature and distributions of oxidants in martian and terrestrial soils the development of field methodologies for the accurate identification and quantification of soil oxidants, especially metal superoxides and peroxides, is needed. In soils, superoxide can exist as adsorbed $O_2^{\bullet-}{}_{ads}$ **(Georgiou *et al.*, 2015)** or as salts of metals with $O_2^{\bullet-}$ in the form of $Me^{+}O_2^{\bullet-}$ (e.g., KO_2, NaO_2) **(Sharma, 2007)**, in the form of $Me^{n+}{-}O_2^{\bullet-}$ such as the ionic complexes of $O_2^{\bullet-}$ with metals of certain minerals and oxides **(Dyrek and Che, 1997; Lunsford, 1973)**. Metal peroxides can exist as salts of metals with the peroxide dianion (O_2^{2-}) bonding either as $Me^{2+}O_2^{2-}$ (e.g., CaO_2, MgO_2) or as $Me^{+}{}_2O_2^{2-}$ (e.g., Na_2O_2, K_2O_2). Metal peroxides can also exist as hydroperoxides (MeO_2H; e.g., of Ti^{4+}, Zr^{4+}, and Ce^{4+}) **(Makarov and Ladelnova, 1961)**. The presence of Mg^{2+}, Ca^{2+}, K^{+} and Na^{+} ions, measured with the Wet Chemistry Laboratory **(Hecht *et al.*, 2009; Kounaves *et al.*, 2010; Quinn *et al.*, 2011)**, may provide the needed counter ions for stabilization of metal superoxides, peroxides and hydroperoxides in the martian soil.

Metal superoxides and peroxides undergo hydrolysis at neutral pH and release the following products (**Fig. 1a,b**):

- Metal superoxides release O_2 (g) and H_2O_2 by the following reactions **(Halliwell and Gutteridge, 1999; Sharma, 2007)**:

  Adsorbed $O_2^{\bullet-}$: $$2O_2^{\bullet-}{}_{ads} + 2H_2O \rightarrow 2OH^{-} + H_2O_2 + O_2 \quad (1)$$

  Me-superoxide salts: $$2Me^{+}O_2^{\bullet-} + 2H_2O \rightarrow 2Me^{+}OH^{-} + H_2O_2 + O_2 \quad (2)$$

  Metal$-O_2^{\bullet-}$ complexes: $$2Me^{n+}{-}O_2^{\bullet-} + 2H_2O \rightarrow 2Me^{n+} + 2OH^{-} + H_2O_2 + O_2 \quad (3)$$

- Metal peroxides/hydroperoxides release H_2O_2 by the following reactions **(Sharma, 2007)**:

  Me-peroxides: $Me^{+}{}_2O_2^{2-} + 2H_2O \rightarrow 2Me^{+}OH^{-} + H_2O_2$ (4)

  $Me^{2+}O_2^{2-} + 2H_2O \rightarrow Me^{2+}(OH^{-})_2 + H_2O_2$ (5)

- Metal hydroperoxides (MeO_2H): $MeOOH + H_2O \rightarrow MeOH + H_2O_2$ (6)

- H_2O_2 released from metal superoxides/peroxides/hydroperoxides can go on to release O_2 (g) by the following conditions, and their association with certain water-soluble soil ionic constituents that are found also on Mars. O_2 may be produced by the alkaline hydrolysis of H_2O_2 (i.e., $2H_2O_2 \rightarrow 2H_2O + O_2$) due to the formation of metal hydroxide ions (reactions 2-6). These hydroxyls could have caused alkalinization of the neutral pH (7.2) of the GEx nutrient. The available evidence suggests an alkaline pH for both 'wet' and 'humid' modes of the Viking GEX experiment **(Klein, 1978; Oyama *et al.*, 1977; Quinn and Orenberg, 1993)**. Another possible mechanism for the decomposition of H_2O_2 to O_2 may have involved its prior reaction with bicarbonates. Bicarbonates may form from the dissolution of soil carbonates and the CO_2 headspace gas that was present in GEX and LR test cells. This process is characterized by the initial formation of a peroxobicarbonate ($MeHCO_4$; Me = Na, K) and its concommitant conversion to the carbonate peroxyhydrate **(Firsova *et al.*, 2005)** as shown by the following reactions:

  $NaHCO_3 + H_2O_2 \leftrightarrow NaHCO_4 \bullet H_2O$ (7)

  $2NaHCO_4 \bullet H_2O \rightarrow Na_2CO_3 \bullet 1.5H_2O_2 + CO_2 + 1.5H_2O + 0.25O_2$ (8)

  Net: $2NaHCO_3 + 2H_2O_2 \leftrightarrow Na_2CO_3 \bullet 1.5H_2O_2 + CO_2 + 1.5H_2O + 0.25O_2$

The lower the H_2O_2 concentration, the more probable is reaction 8 and the more complete is the conversion of the peroxobicarbonate to the carbonate peroxyhydrate. The formation of $MeHCO_4$ peroxocarbonates from bicarbonates and H_2O_2 occurs as a result of nucleophilic substitution of the OH^- by the hydroperoxyl anion (HO_2^-) of H_2O_2 **(Firsova *et al.*, 2005)**. Furthermore, the peroxo carbonate anion (CO_4^{2-}) may decompose H_2O_2 (at excess) via a similar to the perhydroxyl anion reaction path (i.e., $CO_4^{2-} + H_2O_2 \rightarrow CO_3^{2-} + H_2O + O_2$). Calcium carbonate at 2-5 wt. % was detected in soil at the Phoenix Lander site using the Thermal Evolved Gas Analyzer **(Boynton *et al.*, 2009)**. Additionally, a moderately alkaline pH of 7.7 ± 0.5, which is consistent with a carbonate-saturated solution, was measured using the Phoenix Wet Chemistry Laboratory **(Hecht *et al.*, 2009)**. Calcium carbonate can react with water saturated with CO_2 to form the soluble calcium bicarbonate [$CaCO_3 + CO_2 + H_2O \rightarrow Ca(HCO_3)_2$]. O_2 release from peroxide modified TiO_2, a chemical analog to the soil oxidants in Viking biology experiments, has also been previously observed **(Quinn and Zent, 1999)**. When this analogue was exposed to a solution similar to the LR nutrient, labeled CO_2 was released and was attributed to the decomposition of labeled organics by outer-sphere peroxide complexes associated with surface hydroxyl groups of the peroxide-modified TiO_2. The release of O_2 upon humidification was attributed to the formation of more stable inner-sphere peroxide complexes within the peroxide-modified TiO_2, associated with Ti^{4+} cations **(Quinn and Zent, 1999)**. Another possibility is the formation of H_2O_2 and subsequent O_2 production through inner-sphere peroxide complex hydrolysis (by a process similar to that of the hydrolysis of metal peroxides shown in reactions 4 and 5), and its subsequent carbonate- and/or alkaline-catalyzed decomposition to O_2. The formation of a $TiO_3 \cdot 2H_2O$ (peroxide) complex is also possible **(Sharma, 2007)**.

The aforementioned factors affecting stability of soil H_2O_2 must be considered in the determination of metal superoxides and peroxides/hydroperoxides under aqueous conditions. Metal superoxides/peroxides are quantified non-specifically and non-enzymatically by measuring their hydrolysis products O_2 and H_2O_2 **(Sharma, 2007)**. H_2O_2 is measured by acidified $KMnO_4$ or cerium

(IV), and by potentiometric titration with Na-hypochlorite and K-ferricyanide. Moreover, metal peroxides, especially the simpler transition metal-based ones, are determined by the amount of O_2 (½ mol^{-1} peroxyl group) released upon thermal decomposition, provided that the oxidation state of the metal does not change **(Sharma, 2007)**.

In the present study, we developed an enzymatic assay for the detection of metal superoxides (sum of $O_2^{\bullet-}{}_{ads}$, $Me^{+}O_2^{\bullet-}$ and $Me^{n+}{-}O_2^{\bullet-}$) and metal peroxides/hydroperoxides (sum of $Me^{2+}O_2^{2-}$, $Me^{+}{}_2O_2^{2-}$ and MeO_2H) with potential to be extended to field instrumentation for terrestrial and planetary applications. The assay discriminates and quantifies metal superoxides and peroxides/hydroperoxides by measuring the O_2 that is enzymatically released from their dismutation/hydrolysis products; $O_2^{\bullet-}$ and H_2O_2 from the first, and H_2O_2 from the second group. This is achieved at assay conditions that stabilize $O_2^{\bullet-}$ and H_2O_2 and by the following enzymatic reactions **(Halliwell and Gutteridge, 1999)**: (i) the superoxide dismutase (SOD)-catalyzed dismutation of 1 mol $O_2^{\bullet-}$ to ½ mol O_2 and ½ mol H_2O_2, (ii) the oxidized cytochrome c (cyt. c_{ox})-catalyzed conversion of 1 mol $O_2^{\bullet-}$ to 1 mol O_2, and (iii) the catalase (CAT)-decomposition of 1 mol H_2O_2 (from dismutated $O_2^{\bullet-}$ and hydrolyzed metal peroxides/hydroperoxides) to ½ mol O_2**.** Moreover, the OR assay quantifies O_2 also from non-specific soil sources. Simulation of the OR assay was performed in a liquid-phase O_2 electrode with known concentrations of $O_2^{\bullet-}$ and H_2O_2, and in the presence/absence of soils from the Mojave Desert CIMA volcanic field and the Atacama deserts. The assay was further validated on commercial sources of metal superoxides (KO_2) and peroxides (Na_2O_2, CaO_2, MgO_2) in the presence of carbonate and perchlorate ions since both were found on Martian soil. Moreover, γ-radiation experiments were performed to test the cosmic radiation endurance of the OR assay enzymes CAT, SOD and cyt. c_{ox}.

Experimental

The OR assay is based on the release of O_2 from the hydrolysis/dismutation products ($O_2^{\bullet-}$, H_2O_2) of soil metal superoxides and peroxides/hydroperoxides by a certain combination of

enzymatic reactions catalyzed by SOD, CAT and cyt. c_{ox}, followed by the subsequent measurement of these peroxidants by an O_2 electrode. The assay was designed to be used for terrestrial and planetary applications. The enzymatic reaction steps of the OR assay were determined by the following experiments by testing: (a) the functional effectiveness of OR assay enzymes for the effective scavenging of $O_2^{\bullet-}$ (by SOD and cyt. c_{ox}) and (b) the stability of H_2O_2 for its decomposition to O_2 (by CAT), in the presence of perchlorate and carbonate (water-soluble ionic constituents found in Martian soil), and phosphate (H_2O_2-stabilizer and component of the assay buffer); (c) the functional endurance of the OR assay enzymes to cosmic rays upon exposure to γ-radiation; (d) the simulation of the OR assay by indirect testing on commercial analogues of metal superoxides and peroxides, and directly on $O_2^{\bullet-}$ and H_2O_2.

Materials

Sodium peroxide (Na_2O_2; cat no 106563), hydrogen peroxide (H_2O_2, 30%; cat no 107210), perchloric acid (70-72%; cat no 100519**)**, dimethyl sulfoxide (DMSO; cat no 102952), potassium superoxide (KO_2; cat no 814151), and acetonitrile (ACN; cat no 100029) were from Merck, Darmstadt, Germany; dicyclohexano-18-crown-6 ether (CE; cat no A15344), glycine (cat no 43497), and calcium peroxide (CaO_2; cat no 21157) from Alfa Aesar, Germany; homovanillic acid (HVA; cat no H1252), horseradish peroxidase (HRP; cat no H1252), catalase (CAT; cat no C9322) from bovine liver, cytochrome *c* (oxidized; cyt. c_{ox}; cat no 105201) from horse heart, superoxide dismutase (SOD; cat no S7446) from bovine erythrocytes, magnesium peroxide (MgO_2; cat no 433624), titanium (IV) oxide (TiO_2; cat no T8141), *o*-dianisidine dihydrochloride (cat no D3252), diethylene triamine pentaacetic acid (DTPA; cat no D6518) and molecular sieves (MS 4Å, beads 8-12 mesh; cat no 20,806-4) from Sigma-Aldrich, St. Louis, USA; anhydrous sodium carbonate (cat no 13418) from Riedel de Haen AG, Seelze, Germany. All other chemicals used in this study were of the highest purity.

Equipment

Liquid-phase Clark-type O_2 electrode was by Hansatech Instruments Co, England, and controlled by the 'Oxygraph plus' software by the same provider. Sonicator (model UP-50 H from Dr Hielscher GmbH) equipped with a 2-mm-diameter MS2 micro-tip. Fluorescence was measured in a Shimadzu RF-1501 spectrofluorometer set at 10 nm excitation/emission slit width and at low sensitivity, using a quartz microcuvette (internal dimensions 4×4×45 mm). Absorbance was measured in a Shimadzu UV-1800 UV-VIS spectrophotometer. Co-60 source of γ-radiation provided by NCSR 'Demokritos', Attiki, Greece. Illumination apparatus for measuring SOD activity was composed by a circular extra brightness day light fluorescent lamp 22 Watt (i.d. 15 cm, by FUJI Co Ltd, Japan) mounted in a custom made apparatus, where the cuvette (e.g., 1 ml) was centred on a white plastic disk (diam. 6 cm) placed on top of a small electric fan (8x8x2.5 cm) mounted horizontally in the centre of the lamp (in order to keep the cuvette at a steady RT environment).

Description of the enzymatic reactions of the OR assay

The OR assay measures O_2 released from the superoxide and peroxide groups of the controlled hydrolyzed metal superoxides and peroxides/hydroperoxides, respectively. The enzymatic (and accompanying non-enzymatic) reactions involved in the OR assay are presented in **Table 1**.

Testing enzymatic reactions

I. With $O_2^{\bullet-}$ stock solution

$O_2^{\bullet-}$ stock was made (as previously described **(Georgiou *et al.*, 2007)**) by dissolving ~15 mg KO_2 in 1 ml anhydrous (100%) DMSO by sonication (for 30-60 s at 350 W cm^{-2}), followed by the removal of any insoluble matter by centrifugation at 12,000 *g* for 3 min. Anhydrous DMSO was

prepared by mixing 0.1 g molecular sieves ml^{-1} 100% DMSO and letting the mixture stand overnight capped at room temperature (RT). The concentration of the $O_2^{\bullet-}$ stock (~0.7 mM) was determined indirectly by its dismutation (reaction 1) through measurement of the product H_2O_2, which was determined by the *horseradish peroxidase-homovanillic acid (HRP-HVA) assay* **(Ci and Wang, 1990)** as described in a following section. The concentration of the $O_2^{\bullet-}$ stock was also verified by the following *Cytochrome c assay*.

II. With metal superoxide/peroxide dismutation/hydrolysis reactions, their products ($O_2^{\bullet-}$, H_2O_2), and Martian soil ionic constituents

1. Cytochrome *c* assay for $O_2^{\bullet-}$ quantification

The concentration of $O_2^{\bullet-}$ is determined by its reaction with ≥10 molar excess of cyt. c_{ox} (as determined in the following sub-section "*Reaction of $O_2^{\bullet-}$ with cytochrome c and SOD, and the effect of perchlorate*"; typical cyt. c_{ox} concentration range used for this purpose was 20-100 μM, dissolved in 0.25 M K-phosphate buffer, pH 7.2, in ±SOD (45 U ml^{-1}, by adding 10 μl 4.5 KU ml^{-1} SOD stock in 1 ml of the assay buffer before the addition of cyt. c_{ox}). If $O_2^{\bullet-}$ is present the assay will identify it from the SOD-inhibited reduction of cyt. c_{ox} **(Georgiou *et al.*, 2015)**. In principle, this assay measures the concentration of $O_2^{\bullet-}$ from the concentration of cyt. c_{red} that is formed by the equimolar reaction of $O_2^{\bullet-}$ with cyt. c_{ox} (reaction 9). The concentration of cyt. c_{red} is determined from its absorbance at 550 nm (against the absorbance of cyt. c_{ox} and before reacting with $O_2^{\bullet-}$) and its molar extinction coefficient 21 mM^{-1} cm^{-1} at 550 nm **(Van Gelder and Slater, 1962)**.

2. HRP-HVA assay for H_2O_2 quantification

This assay **(Ci and Wang, 1990)** was chosen for its high sensitivity to detect as low as 50 nM H_2O_2 (in a 0.3 ml reaction volume), and is performed as follows: To 0.4 ml 0.25 M K-phosphate buffer, pH 7.2, ~8 μl $O_2^{\bullet-}$ stock solution were added (in order for the dismutation reaction to take

place), and the resulting reaction mixture was split in two 0.2-ml portions: One portion (designated 'sample') is used for measuring the concentration of the formed H_2O_2 (and thus the 2x concentration of the dismutated $O_2^{\bullet-}$ from which it resulted), while to the second portion (designated 'blank') 5 µl 5 KU/ml CAT was added and incubated for 5 min at RT (to destroy the formed H_2O_2). Subsequently, to the resulting sample and blank reaction mixtures 0.05 ml 100 mM glycine buffer (pH 10.2), 0.03 ml 3 mM HVA (made in ddH_2O) and 0.02 ml 250 U ml^{-1} HRP were added and incubated for 15 min in the dark at RT. Fluorescence units (FU) of the sample and the blank were measured at ex/em 316/424 nm. The FU difference between sample and blank was converted to H_2O_2 equivalents from a H_2O_2 standard curve (0-1.5 µM), constructed by the aforementioned procedure.

3. Dismutation/hydrolysis products of commercial analogues of metal superoxides and peroxides

The OR assay detects metal superoxides and peroxides/hydroperoxides by quantifying their hydrolysis products (**Fig. 1a,b**); that is, (i) from the hydrolysis of metal superoxides, the intermediate product $O_2^{\bullet-}$ and its dismutation products H_2O_2 and O_2, and (ii) from the hydrolysis of metal peroxides/hydroperoxides the product H_2O_2. Under the experimental conditions of the OR assay, the formation of H_2O_2 was verified using the following commercially available metal superoxide/peroxide salt analogues; KO_2 for type $Me^{+}O_2^{\bullet-}$ metal superoxides; Na_2O_2 and CaO_2, MgO_2 for type $Me^{+}{}_2O_2^{2-}$ and $Me^{2+}O_2^{2-}$ metal peroxides, respectively. TiO_2 was tested as water-insoluble peroxide control (not belonging to the typical metal peroxide types $Me^{+}{}_2O_2^{2-}$ and $Me^{2+}O_2^{2-}$). An additional reason for this peroxidant analogue selection is that K, Na, Ca, Mg and Ti have been detected in Martian soil **(Rieder *et al.*, 1997)**. According to the general superoxide/peroxide hydrolysis reactions 2-5, these peroxidants are hydrolyzed as follows:

$$2KO_2 + 2H_2O \rightarrow 2KOH + H_2O_2 + O_2 \quad (11)$$

($O_2^{\bullet-}$ in KO_2 undergoes dismutation reaction 1)

$$Na_2O_2 + 2H_2O \rightarrow 2NaOH + H_2O_2 \quad (12)$$

$$CaO_2 + 2H_2O \rightarrow Ca(OH)_2 + H_2O_2 \quad (13)$$

$$MgO_2 + 2H_2O \rightarrow Mg(OH)_2 + H_2O_2 \quad (14)$$

Known amounts of KO_2, Na_2O_2, CaO_2 and MgO_2 were immediately dissolved in 0.25 M K-phosphate buffer, pH 7.2, at RT by continuous stirring to rapidly neutralize the formed alkaline decomposition product MeOH (reactions 11-14). Then, they were treated (i) with an excess concentration of cyt. c_{ox} (this concentration was determined in following sub-section '*Reaction of $O_2^{\bullet-}$ with cytochrome c and SOD, and the effect of perchlorate*'; see also **Fig. 2**), and (ii) in the absence of cyt. c_{ox}. Subsequently, the concentrations of the generated $O_2^{\bullet-}$ and H_2O_2 were measured by the *Cytochrome c* and the *HRP-HVA* assays, respectively, they were converted to concentrations of the corresponding metal superoxides/peroxides, and finally they were compared with their expected concentrations determined by weight (**Table 2**). The following experiments were performed in the absence of DTPA [a metal chelator used in the OR assay in order to protect H_2O_2 from destruction by any soil transition metals such as Fe^{2+} **(Molina and Anchordoquy, 2007)** via the Fenton reaction ($Me^{n+} + H_2O_2 \rightarrow Me^{n+1} + HO^{\cdot} + OH^{-}$) **(Haber and Weiss, 1932)**] in the 0.25 M K-phosphate buffer, because it interferes with the employed *HRP-HVA assay* (data not shown):

i. KO_2: The hydrolysis of KO_2 in the presence of excess cyt. c_{ox} was performed under the following experimental restrictions: The dissolution of 1 mg KO_2 in a volume of 100 ml 0.25 M K-phosphate buffer, pH 7.2, would theoretically result in a 140 μM $O_2^{\bullet-}$ solution, which would then need a 10x higher concentration (1.4 mM) of cyt. c_{ox} to completely scavenge $O_2^{\bullet-}$. This would have resulted in a 140 μM cyt. c_{red} solution with an absorbance (at 550 nm) that would have saturated the detector of the UV-visible spectrophotometer. However, we performed the experiment with the 1 mg KO_2 at 20 μM cyt. c_{ox} (in a 100 ml of the phosphate buffer), where 20 μM $O_2^{\bullet-}$ (out of the 140

μM KO_2) will react with the 20 μM cyt. c_{ox} and the absorbance of the resulting 20 μM cyt. c_{red} at 550 (~0.4) is withing the linear range of the spectrophotometer. The remaining 120 μM $O_2^{\bullet-}$ that escaped cyt. c_{ox} scavenging will dismutate to H_2O_2 (~60 μM according to reaction 1), which was verified by the *HRP-HVA assay*.

ii. Na_2O_2: To 3 ml 0.25 M K-phosphate buffer, pH 7.2, containing ±100 μM cyt. c_{ox}, 1 mg Na_2O_2 (i.e., 12.8 μmol) was dissolved. The concentrations of the H_2O_2 released from the hydrolysis of Na_2O_2 (at a ratio Na_2O_2/H_2O_2 = 1/1, according to reaction 12), and of any $O_2^{\bullet-}$ present (resulting from possible traces of NaO_2 in the Na_2O_2 commercial batch of use) were determined by the *HRP-HVA* and the *Cytochrome c* assay, respectively. Another approach to test whether Na_2O_2 is possibly contaminated with traces of NaO_2, involved the extraction of $O_2^{\bullet-}$ (present as NaO_2) from 7.9 mg Na_2O_2 in 1 ml anhydrous (100%) DMSO (containing 0.2 mM CE to facilitate $O_2^{\bullet-}$ extraction **(Valentine and Curtis, 1975)**) by vortexing for 5 min, followed by centrifugation at 15,000*g* for 5 min (to remove any insoluble matter). The quantification of the $O_2^{\bullet-}$ extracted in the DMSO-CE supernatant was performed by the *Cytochrome c assay* as follows: To 1 ml 0.25 M K-phosphate buffer, pH 7.2, containing 20 μM cyt. c_{ox}, ~50 μl of the $O_2^{\bullet-}$ DMSO-CE extract were added. After 1 min incubation at RT the absorbance difference (sample $A_{550\ nm}$ minus blank $A_{550\ nm}$) was converted to cyt. c_{red} concentration (using its extinction coefficient 21 mM^{-1} cm^{-1} at 550 nm **(Massey, 1959)**), which is equal to the concentration of $O_2^{\bullet-}$ (reaction 9). It should be noted that the *HRP-HVA assay* did not detect any H_2O_2 presence in the DMSO-CE extract of Na_2O_2, possibly because the O_2^{2-} peroxyl group of Na_2O_2 is not dissolved in the aprotic solvent DMSO-CE (data not shown).

iii. CaO_2, MgO_2, TiO_2: The commercial metal peroxides were dissolved at 5 mM in 10 ml 0.25 M K-phosphate buffer, pH 7.2, containing ±20 μM cyt. c_{ox} and ±CAT. Measurements were done over a period of 2 hrs at RT due to the low solubility of these peroxides in water. Water-insoluble TiO_2 was used as negative control for the *HRP-HVA* assay in order to illustrate that the hydrolysis of the water soluble control metal peroxides produces H_2O_2 (using CAT as H_2O_2

identification control). $O_2^{\bullet-}$ was measured by the *Cytochrome c* assay, respectively (using controls CAT and cyt. c_{ox}, respectively).

4. Reaction of $O_2^{\bullet-}$ with cyt. c_{ox} and SOD, and the effect of perchlorate

(i) Reaction of cyt. c_{ox} with $O_2^{\bullet-}$: The excess cyt. c_{ox} concentration to convert $O_2^{\bullet-}$ (from commercial metal superoxides or the $O_2^{\bullet-}$ stock solution) to O_2 (reaction 9) was determined by varying the molar ratio $O_2^{\bullet-}$/cyt. c_{ox}, and measuring the concentration of H_2O_2 (by the *HRP-HVA assay*) that would have been formed by the SOD (45 U ml^{-1})-catalyzed dismutation of $O_2^{\bullet-}$ (10 µl 4.5 KU ml^{-1} SOD stock added in 1 ml of the K-phosphate buffer) if its scavenging by cyt. c_{ox} was not 100% complete. The $O_2^{\bullet-}$/cyt. c_{ox} reaction mixture was composed of 0.4 ml 0.25 M K-phosphate buffer, pH 7.2, containing 200 µM cyt. c_{ox}, to which various amounts of the $O_2^{\bullet-}$ stock solution (8 µl from 1-8 fold stock dilutions made with anhydrous DMSO) were added (**Fig. 2a**). In addition, the concentration of $O_2^{\bullet-}$ that reacted with cyt. c_{ox} to form equimolar cyt. c_{red} was determined by the *Cytochrome c assay*.

(ii) Perchlorate effect on the reaction of cyt. c_{ox} and SOD with $O_2^{\bullet-}$: The effect of perchlorate concentration (110 mM) was tested (i) on the SOD (45 U ml^{-1}) dismutation of 10 µM $O_2^{\bullet-}$ (from the $O_2^{\bullet-}$ stock solution) by 0.25 M K-phosphate buffer, pH 7.2, and (ii) on the 100% effective scavenging of $O_2^{\bullet-}$ by 100 µM cyt. c_{ox} (the required 10 fold molar excess cyt. c_{ox} was determined in the sub-section '*Reaction of cyt. c_{ox} with $O_2^{\bullet-}$*'), given the fact that perchlorate is known to bind to cyt. c_{ox} **(Andersson *et al.*, 1980)**. This was done by measuring the concentration of H_2O_2 generated from the SOD-dismutated $O_2^{\bullet-}$ (the portion of $O_2^{\bullet-}$ that escaped cyt. c_{ox} scavenging) with the *HRP-HVA assay* (**Fig. 2b**). Choosing 110 mM as test perchlorate concentration was based on its concentration (~2.5 mM formed in the 25 ml leaching solution added to 1 cm^3 soil) detected at the Phoenix Mars Lander site **(Hecht *et al.*, 2009)**, by proportionally extrapolating it to the 0.57 ml volume of the M4 nutrient used in the 'humid' mode of the Viking GEX experiment. Although in the 'humid' mode the GEx solution did not contact the soil, 0.57 ml (solution volume) cm^{-3} soil

sample was used to establish the upper perchlorate and carbonate concentration limit for OR assay interference. Sodium perchlorate solution was prepared from a 2.2 M stock prepared by mixing equal volumes 4.4 M perchloric acid and 4.4 M NaOH.

5. Effect of carbonate, phosphate, perchlorate, pH on H_2O_2 stability, and of perchlorate on CAT activity

(i) Carbonate, phosphate and pH effect on H_2O_2 stability: For this experiment, H_2O_2 at 0.5 and 0.1 mM (based on the volumes of liquid used in the Viking GEx experiment) were used. Assuming that the Viking GEx maximum of 775 nmol O_2 **(Oyama *et al.*, 1977)** was formed by the dismutation of 534 nmol $Me(O_2)_2$, the 267 nmol H_2O_2 produced would have resulted in a ~0.1 mM H_2O_2 concentration in the GEx cell. The concentration of carbonates that were mixed with H_2O_2 was derived from the ≥1 mM carbonates detected in the 25 ml leaching solution upon mixing 1 cm^3 soil from the Phoenix Mars Lander site **(Hecht *et al.*, 2009)**. Specifically, the 1 mM concentration of carbonate in the 25 ml leaching solution by proportional extrapolation to the 0.57 ml M4 nutrient volume used in the Viking GEX experiment 'humid' mode corresponds to ~50 mM carbonates, which is actually a saturated carbonate solution. The stability of the tested concentrations of H_2O_2 was determined, (i) in 0.25 M K-phosphate buffer ±50 mM sodium carbonate, and (ii) in 50 mM sodium carbonate (**Fig. 3a,b**). Both experimental conditions were tested over a 3-hr incubation period at RT. The various pH (7-10) of the buffer systems used (K-phosphate, K-phosphate/carbonate and carbonate alone) were appropriately adjusted with NaOH.

(ii) Perchlorate effect on H_2O_2 stability and on CAT activity: The concentrations of perchlorate (25 and 110 mM) mixed with H_2O_2 (0.5 mM and 0.1 mM) were selected based on the perchlorate concentration found on Mars and on the volumes of nutrient used in the GEX experiment, and were prepared as described in the preceeding section. The test solutions of sodium perchlorate and H_2O_2 (made in 0.25 M K-phosphate buffer, pH 7.2) were used to test the H_2O_2

decomposition activity of CAT (at 50 U ml^{-1}) (**Fig. 3c,d**). Stability of H_2O_2 and its decomposition by CAT were measured by the *HRP-HVA assay* over a 3-hr incubation period at RT.

III. Testing the endurance of the OR assay enzymes to cosmic radiation

1. Exposure of CAT, SOD and cyt. c_{ox} to γ-radiation

Various amounts of the ROS enzymes in solid form (CAT at 0.3-1.4 mg, SOD at 25-60 μg, and cyt. c_{ox} at 0.1-0.5 mg) were placed in Eppendorf-type 0.5 ml microcentrifuge tubes and exposed at various γ-radiation doses (0-6 Gy). Radiation dose was also converted to equivalent number of round trips to Mars (0-38), given that a human round-trip mission to Mars with current propulsion systems and comparable shielding would incur a physical radiation dose of about 0.16 Gy and an equivalent dose of about 0.66 Sv **(Zeitlin *et al.*, 2013)**. After exposure, the solid CAT, SOD and cyt. c_{ox} samples were assayed by the following assays. In separate experiments, the same assays were also performed in ±ACN (0-50% v/v) using the unexposed enzyme controls, because the ACN solvent can be used for the extraction of $O_2^{\bullet-}$ from soil samples. The enzymatic assays were performed in 0.25 M K-phosphate buffer, pH 7.2, as its K salt is more water-soluble than its Na salt in the presence of ACN.

2. SOD specific activity

Assay principle: SOD specific activity was studied by a modification of a previous assay **(Lu *et al.*, 2004; Misra and Fridovich, 1977)**, and is based on the SOD-inhibited reduction of oxidized dianisidine (D_{ox}). D_{ox} results from the reaction of its reduced form (D_{red}) by photosensitized riboflavin. This reaction is in competition with the formation of D_{red} from D_{ox} by $O_2^{\bullet-}$, the latter being also generated by the photosensitized riboflavin. Thus, SOD increases the rate of the generation of D_{ox} (absorbing at 500 nm) by scavenging $O_2^{\bullet-}$ (which would otherwise nullify the overall dianisidine photooxidation by reducing the D_{ox} form).

Assay procedure: The γ-ray-exposed and unexposed (control) SOD samples were initially dissolved in 0.1 ml ddH_2O. A volume of the dissolved SOD was then added to a glass tube containing a mixture of 0.5 ml 30 µM riboflavin (in 0.5 M K-phosphate buffer, pH 7.2, made fresh and kept light protected), 0.5 ml solution of ddH_2O with 0-50% ACN, and 20 µl 10 mM *o*-dianisidine dihydrochloride stock (in ddH_2O, stored at 0ºC and light protected). The sample blank was prepared by adding 20 µl ddH_2O in place of *o*-dianisidine. In order to ensure proportional final SOD specific activities, SOD sample volumes varied between 5-10 µl, and at least three samples were tested. For cancelling out any interference from autoxidation of the reagent dianisidine (D_{ox}) in the presence of riboflavin the following additional blanks were prepared (in small glass tubes): Riboflavin reagent blank (RB) is the mixture of 0.5 ml 30 µM riboflavin in 0.5 M K-phosphate buffer, pH 7.2, 0.5 ml ddH_2O (or 100% ACN) and 20 µl ddH_2O; riboflavin/dianisidine reagent blank ($RD_{ox}B$) is the mixture of 0.5 ml 30 µM riboflavin (in 0.5 M K-phosphate buffer, pH 7.2), 0.5 ml ddH_2O (or 100% ACN), and 20 µl 10 mM dianisidine stock. The glass tubes of the sample and corresponding blanks were incubated in the illumination apparatus for 4 min at 26°C, after which their absorbance was measured at 500 nm (by a UV-VIS 1200 Shimadzu spectrophotometer, zeroed with ddH_2O or ACN). The specific activity of SOD [(net D_{ox} absorbance change min^{-1}, or $D_{ox}\Delta A_{500nm}$, min^{-1}) μg^{-1}] was calculated by the formula:

$$(D_{ox}\Delta A_{500nm}/min)\ \mu g^{-1} = \{[(S - SB) - (RD_{ox}B - RB)]/4\ min\}\ \mu g^{-1}\ SOD\ protein$$

Then, the SOD activity factor (ΔA_{500nm}/min) was converted to D_{ox}-based SOD Units, designated as UD_{ox} (where 1 UD_{ox} = 1 µmol D_{ox} min^{-1} in 1 ml assay volume, using the absorption extinction coefficient, ε, for D_{ox}, 7.5 mM^{-1} cm^{-1} at 500 nm **(Chauhan *et al.*, 2013)**), and finally expressed as specific activity (mUD_{ox} μg^{-1} SOD protein; **Fig. 4**), with the protein quantified by a previously reported ultrasensitive assay **(Georgiou *et al.*, 2008)**.

Notes: *I*. Assay blanks RB and SB gave identical absorbance at 500 nm. *II*. The presence of 50% ACN did not affect SOD specific activity.

3. CAT specific activity

Assay principle: CAT specific activity was measured by a modification of the H_2O_2 consumption assay **(Blum and Fridovich, 1983)**. CAT specific activity was determined (in ±ACN up to 50%) by measuring the rate of H_2O_2 concentration decrease photometrically at 240 nm **(Blum and Fridovich, 1983)**, and converted to U μg^{-1} based on the absorbance molar extinction coefficient of H_2O_2 43.6 M^{-1} cm^{-1} at 240 nm **(Martin *et al.*, 1996)**, where 1 U corresponds to the amount of CAT which consumes 1 µmol H_2O_2 min^{-1}.

Assay procedure: The γ-ray-exposed and unexposed (control) CAT samples were initially dissolved in 0.2 ml ddH_2O, and subsequently diluted by ~500 fold. A volume of the diluted CAT was then added to a spectrophotometer quartz cuvette containing a mixture of 0.45 ml 0.5 M K-phosphate buffer, pH 7.2, 50 µl 100 mM H_2O_2 (made in 0.5 M K-phosphate buffer, pH 7.2; H_2O_2 is at final 5 mM, giving A_{240nm} ~0.2), and 0.5 ml ddH_2O with 0-50% ACN. The decrease in H_2O_2 concentration vs time was recorded at 240 nm (against ddH_2O or ACN at the appropriate % concentration). In order to ensure proportional final CAT specific activities, CAT sample volumes varied between 10-20 µl, and at least three samples were tested. The obtained linear slope (an average from at least three CAT solution volumes) is converted to CAT specific activity, expressed in Units (or nmol H_2O_2 min^{-1}) μg^{-1} (**Fig. 4**), with the protein quantified as previously stated **(Georgiou *et al.*, 2008)**.

4. Cyt. c_{ox} specific reducibility

Assay principle: Cyt. c_{ox} readily oxidizes equimolar $O_2^{\bullet-}$ to equimolar O_2 **(Halliwell and Gutteridge, 1999)**. Thus, the functionality of γ-radiation-exposed cyt. c_{ox} can be assessed (compared to the unexposed control) by its specific reducibility (defined as cyt. c_{red} μg^{-1} protein) by a typical organic reductant of cyt. c_{ox} such as ascorbic acid (although at slow rate **(Paciolla and De Gara, 1991)**).

Assay procedure: The γ-ray-exposed and unexposed (control) cyt. c_{ox} samples were dissolved in 0.2 ml 100 mM K-phosphate buffer, pH 7.2, and 30 µl of it were mixed in final 0.3 ml 0.25 M K-phosphate buffer, pH 7.2 (made from a 0.5 M K-phosphate buffer stock) containing 0 to 50% ACN, as to give an absorbance reading (Acyt.c_{ox}) at 550 nm ~0.05-0.1 (~0.055A corresponds to 8 µM cyt. c_{ox}). Subsequently, the 0.3 ml cyt. c_{ox} solution was completely reduced by final 50 mM ascorbic acid (by adding 0.05 ml - or 1.166 diluting - of a 0.35 M ascorbic acid stock made in 0.25 M K-phosphate buffer, pH 7.2). After incubation at RT for 4 min, the absorbance of the resulting cyt.c_{red} solution is corrected for the dilution factor 1.166 and designated as Acyt.c_{red}+Ac_{ox}. The net absorbance of cyt. c_{red}, Acyt.c_{red} [= (Acyt.c_{red}+Ac_{ox}) - (Acyt.c_{ox})] is expressed as pmol cyt. c_{red} μg^{-1} and designated as "specific reducibility" (**Fig. 4**), with the pmol cyt. c_{red} (in 0.3 ml) quantified from Acyt.c_{red} by the molar absorption extinction coefficient of cyt. c_{red} 21.0 mM^{-1} cm^{-1} at 550 nm **(Massey, 1959; Van Gelder and Slater, 1962)**. Protein was quantified by a previously reported assay **(Georgiou *et al.*, 2008)**. It should be noted that (i) cyt. c_{ox} does not remain soluble at ACN concentration above 60%, and (ii) complete reduction of the tested concentration of cyt. c_{ox} by ascorbic acid in the presence of ACN is slower than in its absence (it takes ~4 min to complete).

OR assay

Having verified the individual OR assay enzymatic reactions 1, 9, and 10 on the superoxide/peroxide dismutation/hydrolysis products in the K-phosphate buffer for activity efficiency and for interference by carbonates and perchlorate, the OR assay was simulated in the presence of Atacama and Mojave desert soils as follows:

1. Experimental setup

The simulation was performed in a semi-sealed liquid-phase O_2 electrode. This setup does not allow the testing of the commercial solid analogues of metal superoxides and peroxides, and for the additional reason that their concentration cannot be controlled as not to exceed the upper limit of the

electrode's linear range response. Instead, stock $O_2^{\bullet-}$ and H_2O_2 solutions were used. The electrode reaction chamber was filled with 1 ml K-phosphate-DTPA buffer (0.25 M K-phosphate buffer, pH 7.2, containing 10 mM DTPA) to which the assay reagents were added at constant RT (26˚C). It should be noted that DTPA acts as chelator of any soluble transition metal ions, which can destroy (i) H_2O_2 via the Fenton reaction **(Haber and Weiss, 1932)** and (ii) $O_2^{\bullet-}$ via oxidation, and may also inactivate the OR assay protein reagents CAT and cyt. c_{ox} (iii) via oxidation by the hydroxyl radicals (HO˙) produced via the Fenton reaction, or (iv) by direct inhibition. The simulation was also performed in the presence of 0.1 g Mars analogue soil samples (pulverized rock from the Mojave Desert CIMA volcanic field, and soil from the Atacama Desert Yungay site), added in the electrode chamber and mixed continuously. As $O_2^{\bullet-}$ source, the 0.7 mM $O_2^{\bullet-}$ stock solution (see sub-section '*$O_2^{\bullet-}$ stock solution*') was used. The CAT stock solution used for the OR assay was made fresh (CAT solutions retain their activity only for 2 days when kept frozen at -25˚C), and also the cyt. c_{ox} stock solution.

2. Experiments conducted

For simulating the OR assay enzymatic reactions 1, 9, and 10 in the Clark-type O_2 electrode chamber, the following treatments were performed (**Fig. 5**):

Treatment A (simulation of reaction 1; dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2): To the O_2 electrode chamber, containing 1 ml K-phosphate-DTPA buffer and ±45 units SOD (10 µl of a 4.5 K ml^{-1} SOD stock made in ddH_2O), 70 µl $O_2^{\bullet-}$ stock solution were added (final 50 nM $O_2^{\bullet-}$), and the released O_2 concentration was recorded until reaching a plateau.

Treatment B (simulation of reaction 10; conversion to O_2 by CAT of H_2O_2 released via $O_2^{\bullet-}$ dismutation (spontaneously or by SOD), and H_2O_2 released via peroxide hydrolysis): After recording the first O_2 concentration plateau in *treatment A*, to the resulting reaction mixture 20 U ml^{-1} CAT (10 µl 2 KU ml^{-1} stock) were added, and after

attaining the second O_2 concentration plateau 10 µl 4 mM H_2O_2 (final 40 µM) were added, and the third O_2 concentration plateau was recorded.

Treatment C (simulation of reaction 9; conversion of $O_2^{\bullet-}$ to O_2 by cyt. c_{ox}): The O_2 electrode chamber was refilled with 1 ml K-phosphate-DTPA buffer, which was brought to 0.5 mM (or 0.5 µM) in cyt. c_{ox} by the addition of 25 µl 20 mM cyt. c_{ox} stock, followed by the addition of 70 µl $O_2^{\bullet-}$ stock solution (final 50 nM $O_2^{\bullet-}$), and then the released O_2 concentration plateau was recorded.

3. Mathematical treatment of data

Assuming the presence of $xO_2^{\bullet-}$ and yH_2O_2 mol in the K-phosphate-DTPA buffer (plus zO_2 mol due to unknown sources), this section describes how these peroxidants were calculated from the experiments presented in **Figure 5**. Running the *treatments A*, *B* and *C*, and the calculations are required to offset the presence of the extra zO_2.

The released O_2 concentrations measured by the O_2 electrode during the *A*, *B*, and *C* *treatments* (designated A_{dism}, $A_{dism/CAT}$, and $B_{cyt.c}$, respectively) are given by the following molar equations (based on the molar stoichiometry of each of reactions 1, 9, and 10) as explained in **Figure 5**:

$A_{dism} = ½xO_2 + zO_2$; simplified: $A_{dism} = ½x + z$

$A_{dism/CAT} = ½xO_2 + ¼xO_2 + ½yO_2 + zO_2$; simplified: $A_{dism/CAT} = ¾x + ½y + z$

$B_{cyt.c} = xO_2 + zO_2$; simplified: $B_{cyt.c} = x + z$

The molar concentrations of $xO_2^{\bullet-}$ and yH_2O_2 are then estimated by the following mathematical equations, derived by appropriately combining the molar equations A_{dism}, $A_{dism/CAT}$, and $B_{cyt.c}$ as to cancel out zO_2 (starting by subtracting equation $B_{cyt.c}$ from equation A_{dism} in order to calculate x; then, calculate y by substituting in equation $A_{dism/CAT}$ the sum $½x + z$ by its equal A_{dism}, and also the factor x previously determined):

$x O_2^{\bullet-} = 2(B_{cyt.c} - A_{dism})$

$y H_2O_2 = 2\ A_{dism/CAT} - (B_{cyt.c} + A_{dism})$

Statistical analysis

All data are reported as mean ± standard error (SE) of at least three independent experiments, and were analyzed using the SPSS statistical package (SPSS Inc, 2001, Release 11.0.0, USA). Whenever appropriate, the significance was determined for Student's unpaired *t* tests or ANOVA. A value of $P < 0.05$ was considered to be significant.

Results

Test of the OR assay enzymes for scavenging $O_2^{\bullet-}$ and H_2O_2, and for interference from certain Martian soil constituents

The experiment on the complete scavenging of cyt. c_{ox} with $O_2^{\bullet-}$ by cyt. c_{ox} showed that for the 100% effective (1:1 stoichiometric) reaction of with $O_2^{\bullet-}$ to take place by the OR assay, the molar concentration of cyt. c_{ox} must be at least 10 fold higher than that of $O_2^{\bullet-}$ (**Fig. 2a**). Carbonate was not tested, as it is known to stabilize cytochrome *c* by increase the stability of the closed heme crevice structure **(Osheroff *et al.*, 1980)**. Another concern for the feasibility of this reaction was the known binding of perchlorate to cyt. c_{ox} **(Andersson *et al.*, 1980)**. However, the test of perchlorate on the reaction of $O_2^{\bullet-}$ with cyt. c_{ox} showed that this oxidant does not affect the 100% efficient scavenging of $O_2^{\bullet-}$ by cyt. c_{ox} when the latter is used at $\geq$10 fold molar excess (**Fig. 2b**). Perchlorate did not interfere with the SOD-catalyzed dismutation of $O_2^{\bullet-}$ (**Fig. 2b**).

Stability of H_2O_2 and CAT activity were tested in the presence of carbonates and phosphates versus pH and perchlorate (the latter at pH 7.2) (**Fig. 3**). H_2O_2 was stable in the presence of phosphates plus carbonates or in phosphates alone (**Fig. 3a**). Carbonates alone decomposed H_2O_2 by ~20% only at pH 10 and after incubation for 3 hrs (**Fig. 3b**). Perchlorate (even at 110 mM) did not affect the stability of H_2O_2, nor did it affect CAT activity, both at pH 7.2 (**Fig. 3c, d**).

Endurance of the OR assay enzymes CAT, SOD and cyt. c_{ox} to cosmic radiation

Solid SOD, CAT and cyt. c_{ox} enzymatic function was tested upon exposure to γ-radiation (**Fig. 4a**), and also in 0-50% ACN; **Fig. 4b**). Both SOD and cyt. c_{ox} retained their activities up to ~7 Gy, and also up to 50% ACN. CAT specific activity was unaffected up to ~3 Gy, while up to 6 Gy decreased linearly to 40% (of its unexposed activity). On the other hand, CAT specific activity was decreased by ACN by a fold factor that was numerically near equal to the % concentration of ACN.

Experimental simulation of the OR assay

The OR assay was experimentally tested by an indirect and a direct methodology. Using the indirect method, the OR assay was performed by two methodological approaches:

(i) The $O_2^{\bullet-}$-specific reaction of cyt. c_{ox} with the metal superoxide analogue KO_2 by measuring the decrease in H_2O_2 formation that a certain quantity of cyt. c_{ox} would have caused. This was also used to test the NaO_2 traces present in the metal peroxide analogue Na_2O_2.

(ii) The quantification of H_2O_2 (using CAT as H_2O_2 negative identification control) generated by the hydrolysis of the metal superoxide analogue KO_2 and the metal peroxide analogues Na_2O_2, CaO_2 and MgO_2 (**Table 2**).

The quantification data on generated H_2O_2 are in agreement with the H_2O_2 concentrations predicted by the stoichiometry of the corresponding reactions 11-14. As expected, the control for water-insoluble metal peroxides TiO_2 did not liberate H_2O_2 (data not shown) because it does not belong to the metal peroxide typical types (i.e., $Me^{+}{}_2O_2^{2-}$ and $Me^{2+}O_2^{2-}$). This result due to the insolubility of TiO_2 in water is supported by the high sensitivity for H_2O_2 (15 pmol) of the employed fluorometric *HRP-HVA assay*.

The OR assay was experimentally tested using the direct methodology by performing the assay with known concentrations $O_2^{\bullet-}$ and H_2O_2 in a Clark-type O_2 electrode (**Fig. 5**). Stock $O_2^{\bullet-}$

and H_2O_2 were used because the experimental set up is not suited for the addition of solid samples of metal superoxides and peroxides during released O_2 measurements. Tests using the direct methodology were also performed in the presence of small amounts of pulverized rock from the Mojave Desert Cima volcanic field and soil sand from the Atacama Yungay station. The metal ion chelator DTPA **(Sillanpää, 1996)** was also included in the assay K-phosphate buffer because soil samples may release metal ions upon H_2O-wetting, which, in turn, may inhibit the OR assay enzymes SOD, CAT and cyt. c_{ox}, and also decompose $O_2^{\bullet-}$ and H_2O_2. However, when testing the assay in the presence of the desert samples in the absence of DTPA, the results were not affected at the maximum 0.1 g sample quantity that was used in order not to damage the O_2 electrode. The released O_2 concentrations (A_{dism}, $A_{dism/CAT}$, $B_{cyt.c}$; **Fig. 5**) during the methodologically direct simulation of the OR assay were those predicted by the stoichiometry of each of the assay reactions 1, 9 and 10. The simulation of the OR assay produced same results regardless of the presence of soil samples from Atacama and Mojave Deserts (data not shown).

Discussion

Enzymes and reaction conditions of the OR assay

Cytochrome *c*: The key reaction that allows the OR assay to discriminate between metal superoxides and peroxides/hydroperoxides is the specific scavenging of $O_2^{\bullet-}$ by cyt. c_{ox} to produce equimolar O_2 (reaction 9). The scavenging action of cyt. c_{ox} towards $O_2^{\bullet-}$ is based on the second order rate constant, 1.1×10^6 M^{-1} s^{-1}, of the involved reaction at pH 7.0 **(Butler *et al.*, 1982; Koppenol *et al.*, 1976)**. Compared to the rate constant (~2×10^5 M^{-1} s^{-1} at pH 7.0) of the competing dismutation reaction of $O_2^{\bullet-}$ with H_2O **(Halliwell and Gutteridge, 1999)**, $O_2^{\bullet-}$ reacts with cyt. c_{ox} ~6 fold faster than with H_2O at pH 7.0. We found that at this pH $O_2^{\bullet-}$ is 100% effectively scavenged by cyt. c_{ox} when present in the assay reaction at a ≥10 fold higher molarity (**Fig. 2a**). With the OR assay pH set at 7.2, the $O_2^{\bullet-}/HO_2^{\cdot}$ molar ratio at this pH is ~200 (using pK_a 4.88), which ensures that the superoxide radical exists predominantly in its anionic form when reacting with cyt. c_{ox}

(Halliwell and Gutteridge, 1999; Holleman and Wiberg, 2001). Perchlorate ions at concentrations found on Mars do not affect the scavenging reaction (**Fig. 2b**). Assuming the presence of perchlorate on Mars as metal salt, the neutral pH of the K-phosphate buffer used in the OR assay will retain perchlorate in its salt form. This, in turn, will not allow the protonation reaction of $O_2^{\bullet-}$ by perchloric acid to take place (i.e., $HClO_4 + O_2^{\bullet-} \rightarrow HO_2^{\cdot} + ClO_4^{-}$) **(Sawyer and Valentine, 1981)**. Thus, perchlorate will not change the concentration of the $O_2^{\bullet-}$ fraction in its reaction with cyt. c_{ox}, and, thus, it will not interfere with the OR assay in this respect.

Superoxide dismutase: Another key reaction of the OR assay key is the dismutation of $O_2^{\bullet-}$ by SOD in the assay K-phosphate buffer to release H_2O_2 and O_2 (reaction 1). This reaction is not affected by carbonates **(Vesela and Wilhelm, 2002)**, nor it is affected by perchlorate ions at concentrations found on Mars (**Fig. 2b**). Even if perchlorate existed in Martian soil in acid form, the concentration of the pH neutral assay K-phosphate buffer would have inhibited the conversion of $O_2^{\bullet-}$ to its protonated form $HO_2^{\cdot}$ by $HClO_4$. But even if some of the superoxide radical existed as conjugated acid ($HO_2^{\cdot}$), its dismutation would have still proceed according to the reaction $HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2$ much faster than the 4-substrate reaction 1 **(Halliwell and Gutteridge, 1999; Sawyer and Valentine, 1981)**.

Catalase and H_2O_2 substrate stability: The third important reaction of the OR assay is the conversion of H_2O_2 (dismutation and hydrolysis product of metal superoxides and peroxides/hydroperoxides, respectively) to O_2 by CAT (reaction 10). H_2O_2 stability and CAT activity were tested in the presence of carbonates and phosphates versus pH and perchlorate ($NaClO_4$ at pH 7.2) (**Fig. 3**). The rate of the non-enzymatic decomposition of H_2O_2 depends on its concentration, temperature and pH (it occurs more rapidly in alkaline pH). H_2O_2 stability is incompatible with many inorganic substances that catalyze its decomposition, including most of the transition metals and their compounds. Common catalysts include MnO_2, silver, platinum, lead, ruthenate, and RuO_2, which decompose H_2O_2 to O_2 in alkaline solution **(Venkatachalapathy *et al.*, 1999)**. The K-phosphate buffer of the OR assay is supplemented with the metal chelator DTPA (at

10 mM) in order to enhance the stability of H_2O_2 by preventing (chelating) transition metals to participate in the Fenton reaction **(Molina and Anchordoquy, 2007; Sillanpää, 1996)**. Moreover, DTPA would protect the enzymes of the OR assay from water-soluble metal inhibitors possibly present in soils.

Perchlorate had no effect on H_2O_2 stability and on CAT activity under the pH-neutral conditions of the OR assay (**Fig. 3c,d**), possibly counteracting the known acid-decomposition of H_2O_2 by perchloric acid to $HO^{\cdot}$ and peroxonium ($HOO^{\cdot}$) radical **(Bouchal *et al.*, 1980)**. This is also in agreement with the previous finding that the rates of binding and association constants (thus, the inhibitory action) of perchlorate with CAT (at concentrations similar to those found on Mars topsoil) decrease inversely with pH (reaching a minimum at pH ≥7.0), although perchlorate is known to form a reversible and enzymatically inactive complex with CAT (from human red blood cells **(Aviram, 1980)**). Moreover, CAT activity is not inhibited substantially with either of the metal ions Na^+, K^+, Mg^{2+} detected on Mars **(Hecht *et al.*, 2009)**. On the other hand, Ca^{2+} (also detected on Mars) enhances CAT activity alone, while it restores it in the presence of the others **(Akyilmaz and Kozgus, 2009; Federici *et al.*, 1996)**.

Carbonate besides not affecting CAT activity **(Burge, 1920)** had no effect on the stability of H_2O_2 at the neutral pH of the OR assay, while it destabilized it (decreased it by ~20%) at pH 10 and after 3-hr incubation (**Fig. 3b**). At pH 10, carbonates also exist as $NaHCO_3$ (due to their pK_{a2} 10.33), which could account for a 25% decomposition of H_2O_2 possibly due to the sum of the reactions 7 and 8. Therefore, the OR assay must include rapid mixing of its reagents with soil in order to eliminate the formation of localized high alkaline pH spots which may decompose H_2O_2. Mixing will also ensure the rapid release of O_2 due to the more homogenous solubilization and dismutation/hydrolysis of soil metal superoxides and peroxides/hydroperoxides.

H_2O_2 was stabilized both by phosphates alone and being together with carbonates (**Fig. 3a**). This is in agreement with the known stabilizing role of phosphates on H_2O_2, via the formation of corresponding H_2O_2 complexes **(Kelly *et al.*, 2003)**. Moreover, carbonates are expected on Mars to

be at their high oxidation state, at which they are practically insoluble in water (e.g., solubility of $CaCO_3$ and $MgCO_3$ is 0.15 and 1.2 mM, respectively, at 25°C). The buffered K-phosphates of the OR assay would precipitate any soluble carbonates by forming water-insoluble carbonate/phosphate complexes. With the Martian dust and the Phoenix soil site containing an assumed minimum level of 2-5% carbonates **(Bandfield *et al.*, 2003; Boynton *et al.*, 2009)**, 1 g soil in a 1 ml reagent volume of the OR assay would correspond to ~0.5 M carbonates of high oxidation state, which would be practically insoluble in the assay K-phosphate buffer.

Effect of cosmic radiation and ACN on the OR assay enzymes

Another consideration for the OR assay is whether its enzymatic components SOD, CAT, and cyt. c_{ox} would have been be functional upon exposure to cosmic radiation if this assay were the basis for the development of an instrument to search for $O_2^{\bullet-}$ and H_2O_2 in distant planets such as Mars or the Jupiter's moon Europa. To answer this question cosmic radiation simulation experiments were performed, where solid SOD, CAT and cyt. c_{ox} were exposed to γ-radiation at a dose range comparable to the one they would receive during a space mission e.g., to Mars or Europa, and their functional activities were compared to unexposed controls (**Fig. 4a**). Same activities were also determined for these enzymes in ACN (**Fig. 4b**). This solvent can be used to extract $O_2^{\bullet-}$ from soil samples and purge out from soil any unknown source trapped O_2 in order to be accounted for in the field variations of the OR assay. SOD and cyt. c_{ox} retained functional activity after exposure to a γ-radiation dose of 6 Gy (an equivalent to the cosmic radiation dose received from 38 round trips to Mars **(Zeitlin *et al.*, 2013)**), while CAT specific activity was unaffected up to ~3 Gy after which it decreased linearly to 40% (of its unexposed activity) at 6 Gy. Both SOD and cyt. c_{ox} retained their activities unaffected iinup to 50% ACN, while that of CAT decreased by a fold comparable to the concentration of ACN. For example, at the maximum tested 50% ACN concentration, the initial CAT specific activity of ~3 U μg^{-1} decreased by 50 fold. This practically means that if an OR assay-based instrument requires e.g., 3 U μg^{-1} CAT to function

properly, it should be supplied with 50x CAT (150 U μg^{-1}) when using ACN as assay reagent at maximum 50% (v/v).

Simulation of the ROS assay

The OR assay was simulated by its enzymatic reactions 1, 9, and 10 by which the hydrolysis products ($O_2^{\bullet-}$, H_2O_2) of soil superoxides/peroxides are specifically converted to O_2, the recorded (by an O_2-electrode) values of which are converted to specific concentrations of total metal superoxides and total peroxides/hydroperoxides, based on the stoichiometry of each of the involved enzymatic reactions (**Fig. 5**). The reactivities of the enzymes of the OR assay were simulated towards $O_2^{\bullet-}$, H_2O_2, and commercial metal superoxide and peroxide analogues under assay conditions that stabilize H_2O_2 (high ionic strength K-phosphate buffer, pH 7.0, and the metal chelator DTPA), and in the presence of Mars-like soils from the deserts Mojave and Atacama.

The potential of the OR assay for a field-deployable instrument

The usefulness of the OR assay can be extended to the search of possible metal supero/peroxidant cycles in terrestrial and extraterrestrial ecosystems, especially in light of the recent discovery of the photo-generation of $O_2^{\bullet-}$ and H_2O_2 in Mars-like desiccated Atacama and Mojave Desert soils and its expected occurrence on Mars and other planetary bodies **(Georgiou *et al.*, 2015)**. Future field implementation of the OR assay may involve a reaction chamber scheme with a O_2-sensor (preferably solid state) to monitor the release of peroxidant O_2 from a soil sample during interaction (under constant stirring) with SOD, cyt. c_{ox} and CAT (as simulated in **Fig. 5**) in the following procedures (**Fig. 6**): (i) *Procedure A* involves treatment of soil sample with SOD in the K-phosphate-DTPA buffer, followed by treatment with CAT; (ii) *Procedure B* involves soil treatment either with cyt. c_{ox} alone, or with cyt. c_{ox} followed by CAT. Specifically:

In *procedure A*, soil metal peroxides/hydroperoxides (group of $Me^{+}{}_2O_2^{2-}$ / $Me^{2+}O_2^{2-}$ / MeO_2H) and superoxides (group of $O_2^{\bullet-}{}_{ads}$ / $Me^{+}O_2^{\bullet-}$ / $Me^{n+}{-}O_2^{\bullet-}$) undergo the following enzymatic

conversions: (i) upon addition of SOD, these soil peroxidant groups are hydrolyzed and dismutated (dism) to yield H_2O_2 and $H_2O_2 + O_2$, respectively, and the released O_2 gas plateau concentration rate is recorded by the O_2 sensor (designated A_{dism}); (ii) subsequently, CAT is added in order to decompose H_2O_2 (generated by the hydrolysis of both groups) to O_2, and then the total released O_2 plateau concentration rate is also recorded (designated $A_{dism/CAT}$, as it includes A_{dism}).

In *procedure B*, cyt. c_{ox} is added in excess (premixed in the K-phosphate-DTPA buffer) as to rapidly scavenge $O_2^{\bullet-}$ (released from the hydrolysis of the soil metal superoxides group) to convert it to equimolar O_2, the concentration rate plateau of which is recorded (designated $B_{cyt.c}$). In addition, CAT is added in *procedure B* after recording $B_{cyt.c}$ in order to decompose H_2O_2 (generated by the hydrolysis of the soil metal peroxides/hydroperoxides group) to O_2, and the total released O_2 plateau concentration rate is recorded (designated as $B_{cyt.c/CAT}$, as it includes $B_{cyt.c}$).

Finally, the recorded A_{dism}, $A_{dism/CAT}$ and $B_{cyt.c}$ or $B_{cyt.c/CAT}$ oxygen plateau concentration rate values are used to calculate the concentrations of the groups of metal peroxides/hydroperoxides and superoxides by appropriated mathematical equations, which are derived from the molar stoichiometry of each of the reactions 1, 9, and 10 as follows:

After equating the moles of total superoxides ($O_2^{\bullet-}{}_{ads}/Me^{+}O_2^{\bullet-}/Me^{n+}{-}O_2^{\bullet-}$) to $xO_2^{\bullet-}$, the moles of total peroxides ($Me^{+}{}_2O_2^{2-}/Me^{2+}O_2^{2-}/MeO_2H$) to yH_2O_2 (from reactions 1-3 and 4-6, respectively, and **Fig. 1**), and the moles of O_2 released from unknown sources to zO_2, the released moles of O_2 recorded as A_{hydr}, A_{CAT} and $B_{cyt.c}$, $B_{cyt.c/CAT}$ by the O_2 sensor are stoichiometrically defined by the following set of molar equations:

$A_{dism} = ½xO_2$ (derived from the dismutation of the $O_2^{\bullet-}$ moles that are contained in the group of the metal superoxides $O_2^{\bullet-}{}_{ads}/Me^{+}O_2^{\bullet-}/Me^{n+}{-}O_2^{\bullet-}$; it is based on the stoichiometry of the dismutation reaction 1: $xO_2^{\bullet-} + xH^{+} \rightarrow ½xH_2O_2 + ½xO_2$) and zO_2); simplified: $A_{dism} = ½x + z$

$A_{dism/CAT}$ = sum of $½xO_2$ and zO_2 (formed as in A_{dism}) plus sum of $¼xO_2$ and $½yO_2$ [$¼xO_2$ is generated from the CAT-decomposition of the $½xH_2O_2$ moles that are released from the

hydrolysis of the group $O_2^{\bullet-}{}_{ads}/Me^{+}O_2^{\bullet-}/Me^{n+}{-}O_2^{\bullet-}$; ½$y$$O_2$ is generated from the CAT-decomposition of the $y$$H_2O_2$ moles, which are derived from the hydrolysis of the metal peroxides group $Me^{+}{}_2O_2^{2-}/Me^{2+}O_2^{2-}/MeO_2H$; both CAT-decompositions take place according the stoichiometry of reaction 10: $2H_2O_2 \rightarrow 2H_2O + O_2$); simplified: ¾$x$ + ½y + z

$B_{\text{cyt.}c}$ = the sum of $x$$O_2$ (according the stoichiometry of reaction 9: $O_2^{\bullet-}$ + cyt. $c_{ox} \rightarrow O_2$ + cyt. c_{red}) and $z$$O_2$; simplified: $B_{\text{cyt.}c} = x + z$.

$B_{cyt.c/CAT}$ = the sum of $x$$O_2$ (according the stoichiometry of reaction 9), $z$$O_2$ and ½$y$$O_2$ (derived as explained in the molar equation $A_{dism/CAT}$); simplified: $B_{cyt.c/CAT} = x + ½y + z$

Because soil treatment in *procedure A* includes two separate steps (measuring A_{dism} and $A_{dism/CAT}$) and *procedure B* can be performed by two alternative ways (measuring $B_{cyt.c}$ or $B_{cyt.c/CAT}$), the above molar equations can be analyzed in three independent O_2 measuring sets of molar equations: 1st set accounting for measurements A_{dism}, $A_{dism/CAT}$, $B_{cyt.c}$; 2nd set accounting for measurements A_{dism}, $B_{cyt.c}$, $B_{cyt.c/CAT}$; 3rd set accounting for measurements A_{dism}, $A_{dism/CAT}$, $B_{cyt.c/CAT}$. Such mathematical analysis produces three independent methods for the derivation of (i) the mol concentration ($x$$O_2^{\bullet-}$) of the metal superoxide group ($O_2^{\bullet-}{}_{ads}$ / $Me^{+}O_2^{\bullet-}$ / $Me^{n+}{-}O_2^{\bullet-}$), (ii) the mol concentration ($y$$H_2O_2$) of the metal peroxide/hydroperoxide group ($Me^{+}{}_2O_2^{2-}$ / $Me^{2+}O_2^{2-}$ / MeO_2H), and (iii) the mol concentration of O_2 ($z$$O_2$) from other unknown sources. The three methods are derived as follows:

Method I: By appropriate combination of the 1st set of equations as to initially cancel out $z$$O_2$ (starting with the subtraction of equation A_{dism} from equation $B_{cyt.c}$ in order to derive the equation of $x$$O_2^{\bullet-}$), and then starting with equation $A_{dism/CAT}$ (where the sum ½x + z is substituted by its equal A_{dism}, and the x factor by its previously determined equation in order to derive the equation of $y$$H_2O_2$), the $x$$O_2^{\bullet-}$, $y$$H_2O_2$ and $z$$O_2$ moles in the soil are estimated by the following mathematical equations:

$$x O_2^{\bullet-} = 2(B_{cyt.c} - A_{dism}) \qquad \text{(eq. 1)}$$

$yH_2O_2 = 2A_{dism/CAT} - (B_{cyt.c} + A_{dism})$ (eq. 2)

$zO_2 = A_{dism} - ½x = A_{dism} - 2(B_{cyt.c} - A_{dism})/2 = 2A_{dism} - B_{cyt.c}$

Assuming the presence in soil of only metal superoxides and peroxides/hydroperoxides, their mol concentration by *method I* is given by the following respective equations:

$xO_2^{\bullet-} = 2(B_{cyt.c} - A_{dism}) = 4(A_{dism/CAT} - A_{dism})$, or $A_{dism} = 2A_{dism/CAT} - B_{cyt.c}$

$yH_2O_2 = 2(A_{dism/CAT} - B_{cyt.c}) = 2(A_{dism/CAT} - A_{dism})$, considering that $A_{dism} = B_{cyt.c}$

Method II: After combining the 2nd set of equations in order to cancel out zO_2 (starting by substituting factor $x + z$, in the equation $B_{cyt.c/CAT}$, with its equal $B_{cyt.c}$ in order derive the equation of yH_2O_2, and then subtracting the equation A_{dism} from the equation $B_{cyt.c}$ in order to derive the equation of $xO_2^{\bullet-}$), the $xO_2^{\bullet-}$, yH_2O_2 and zO_2 moles in the soil are estimated by the following mathematical equations:

$xO_2^{\bullet-} = 2(B_{cyt.c} - A_{dism})$ (eq. 3)

$yH_2O_2 = 2(B_{cyt.c/CAT} - B_{cyt.c})$ (eq. 4)

$zO_2 = A_{dism} - ½x = 2A_{dism} - B_{cyt.c}$

Assuming that soil contains only metal superoxides and peroxides/hydroperoxides, their concentration by *method II* is given by the following respective equations:

$xO_2^{\bullet-} = 2(B_{cyt.c} - A_{dism})$, and also $B_{cyt.c/CAT} = B_{cyt.c}$

$yH_2O_2 = 2(B_{cyt.c/CAT} - B_{cyt.c})$, and also $B_{cyt.c} = A_{dism}$

Method III: After combining the 3rd set of equations in order to cancel out zO_2 (starting by solving equation $B_{cyt.c/CAT}$ for $½y$ and then substituting this in equation $A_{dism/CAT}$ in order to derive the equation of $xO_2^{\bullet-}$, and then starting with equation $A_{dism/CAT}$, where the sum $½x + z$ is substituted by its equal A_{dism} in order to derive the equation of yH_2O_2), the $xO_2^{\bullet-}$, yH_2O_2 and zO_2 moles in the soil are estimated by the following mathematical equations:

$xO_2^{\bullet-} = 4(B_{cyt.c/CAT} - A_{dism/CAT})$ (eq. 5)

$yH_2O_2 = 4A_{dism/CAT} - 2(B_{cyt.c/CAT} + A_{dism})$ (eq. 6)

$zO_2 = A_{dism} - ½x = A_{dism} - 4(B_{cyt.c/CAT} - A_{dism/CAT})/2 = A_{dism} - 2(B_{cyt.c/CAT} - A_{dism/CAT})$

Assuming that only metal superoxides and peroxides/hydroperoxides are present in the soil, their concentration by *method III* is given by the following respective equations:

$xO_2^{\bullet-} = 2(B_{cyt.c/CAT} - A_{dism}) = 4(A_{dism/CAT} - A_{dism})$, and also $A_{dism} = 2A_{dism/CAT} - B_{cyt.c/CAT}$

$yH_2O_2 = 2(B_{cyt.c/CAT} - A_{dism})$, and also $A_{dism/CAT} = B_{cyt.c/CAT}$

As noted, the OR assay quantifies also O_2 released from other unknown sources. However, the OR assay will not discriminate the H_2O_2 from the hydrolysis of metal superoxides/peroxides from any free H_2O_2 existing in mineral pore spaces or derived from other sources. The accurate quantification of metal superoxides and peroxides/hydroperoxides by the OR assay assumes that their hydrolysis product H_2O_2 remains stable during measurement in order to be specifically converted to O_2 by CAT. Even if such H_2O_2 could be possibly converted to O_2 by other factors than CAT (e.g., by soil high concentration in carbonates and high alkalinity) during measurement by either *method* (*I*, *II, III*), this will not affect the accurate determination of metal superoxide and peroxide concentrations because CAT will convert H_2O_2 to O_2 at a much faster rate. Moreover, if *methods I*, *II* were applied, the estimation of metal superoxides by their identical equations 1, 3 does not depend on the O_2 released from the decomposition of H_2O_2 (as the additional release of O_2 in *procedures* A_{dism} and $B_{cyt.c}$ is cancelled out by the subtraction factor $C_{\text{cyt.}c} - A_{dism}$). Moreover, it has been noted that such interferences could be also eliminated by the use of the metal chelator DTPA and the phosphate component of buffering reagent used in the OR assay.

The principle of the OR assay be used for the development of an instrument for the detection of planetary $O_2^{\bullet-}$ and H_2O_2 with the following considerations: 1. OR assay enzymes SOD, CAT and cyt. c_{ox} are used in excess (SOD and CAT are sufficient in quantities of few activity units, and cyt. c_{ox} at >10 fold higher molar concentration than $O_2^{\bullet-}$). 2. The enzymes are stored (at -20°C for long-term storage) separate from their solvents, and mixed right before use. This can be accomplished by storing them in three separate e.g. reagent crucibles (analogous to those used in the Wet Chemistry Laboratory of the 2007 Phoenix Mars Scout Lander mission **(Kounaves *et al.*, 2009)**) or

commercially available dual chamber prefillable syringes (one chamber for storing the enzyme and one for its solvent, to be mixed upon piston movement), and their sequential dispensing in the soil chamber of the instrument under continuous mixing of its reagents with the soil sample. 3. The instrument can use solid state optical sensing O_2-electrodes of high sensitivity. Such electrodes are commercially available, are based on the luminescence quenching by O_2, and are sensitive enough (typical detection limit 0.002% or 1 ppb for aqueous/gaseous O_2) to measure O_2 at much lower concentrations (~1 nmol O_2 cm^{-3}) than that (775 nmol) detected by the GEX experiment **(Oyama *et al.*, 1977)**.

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Authors' Disclosure Statement

No competing financial interests exist.

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Tables

Table 1: Reactions of the OR assay	
Metal superoxides ($O_2^{\bullet-}{}_{ads}$, $Me^+O_2^{\bullet-}$, $Me^{n+}{-}O_2^{\bullet-}$); additional details for their hydrolysis/dismutation reaction mechanisms are presented in **Fig. 1a**.	**1.** Dissociation reaction (e.g., of $Me^+O_2^{\bullet-}$): $Me^+O_2^{\bullet-}$ (in H_2O) → $O_2^{\bullet-}$ + Me^+ *Note*: Stable solution of $O_2^{\bullet-}$ is obtained by dissociation of $Me^+O_2^{\bullet-}$ (e.g., KO_2) in DMSO (see following section "$O_2^{\bullet-}$ stock solution") **2.** Release of O_2 via enzymatic dismutation of $O_2^{\bullet-}$ by SOD: $2O_2^{\bullet-} + 2H_2O \rightarrow 2OH^- + H_2O_2 + O_2$ (same as reaction 1) *Note*: The spontaneous dismutation of $O_2^{\bullet-}$ by H_2O has a rate constant ~$2x10^5$ M^{-1} s^{-1}, while that of SOD is $6.4x10^9$ M^{-1} s^{-1} **(Gray and Carmichael, 1992)** (32,000 fold faster) **3.** Release of O_2 via enzymatic oxidation of $O_2^{\bullet-}$ by excess cyt. c_{ox}: $O_2^{\bullet-}$ + cyt. c_{ox} → cyt. c_{red} + O_2 (reaction 9) *Note*: Excess cyt. c_{ox} prevents competing dismutation of $O_2^{\bullet-}$ by H_2O **(Halliwell and Gutteridge, 1999)** **4.** Base formation reaction: $Me^+ + OH^- \rightarrow MeOH$
Metal peroxides and hydroperoxides ($Me^+{}_2O_2^{2-}$, $Me^{2+}O_2^{2-}$, MeOOH); additional details in **Fig. 1b**.	**1.** Dissociation reaction (e.g., of $Me^+{}_2O_2^{2-}$): $Me^+{}_2O_2^{2-}$ (in H_2O) → O_2^{2-} + $2Me^+$ **2.** Hydrolysis reaction of O_2^{2-}: $O_2^{2-} + 2H_2O \rightarrow 2OH^- + H_2O_2$ **3.** Base formation reaction: $2Me^{2+} + 2OH^- \rightarrow 2Me^+OH$
H_2O_2 released by the hydrolysis of metal superoxides, peroxides and hydroperoxides	Release of O_2 via enzymatic decomposition of H_2O_2 by CAT **(Halliwell and Gutteridge, 1999)**: $2H_2O_2 \rightarrow 2H_2O + O_2$ (reaction 10)

Table 2. Hydrolysis products of commercial analogues of metal superoxides and peroxides				
Metal oxidants	Metal oxidant concentration (based on metal oxidant weight used)	$O_2^{\bullet-}$ concentration (by the *Cytochrome c assay*)	H_2O_2 concentration (by the *HRP-HVA assay*)	Metal oxidant concentration (determined by the *Cytochrome c* and the *HRP-HVA* assay)
KO_2	140 μM	19 ± 3 μM	58 ± 7 μM	137 ± 15
Na_2O_2	4.27 mM	10[a] ± 2 μM	4.2 ± 0.1 mM	4.2 ± 0.1 mM
CaO_2	5 mM	~0[a]	4.8 ± 0.3 mM	4.8[b] ± 0.3 mM
MgO_2	5 mM	~0[a]	5.1 ± 0.2 mM	5.1[b] ± 0.2 mM
TiO_2	5 mM	~0[a]	~0	~0[b]

[a]The concentration 10 μM $O_2^{\bullet-}$ determined from 1 mg (or 12.8 μmol) Na_2O_2 dissolved in 3 ml K-phosphate-cyt. c_{ox} buffer, correspond to 30 nmol $O_2^{\bullet-}$ (presumably released by 30 nmol NaO_2) or to 0.23 mol NaO_2 per 100 mol Na_2O_2. Alternatively, the determined (also by the *Cytochrome c assay*) 0.24 mM $O_2^{\bullet-}$ extracted in 1 ml CE-DMSO solvent from 7.9 mg (or ~100 μmol) Na_2O_2, correspond to 0.24 μmol NaO_2, or to 0.24 mol NaO_2 per 100 mol Na_2O_2 (or 0.17% w/w NaO_2). These two different experimental approaches verify that the commercial Na_2O_2 batch used in the present study contains a minor quantity of NaO_2. No reduction of cyt. c_{ox} was observed with CaO_2, MgO_2, and TiO_2, suggesting that there are no metal superoxide impurities in these peroxides.

[b]These concentrations were attained after 0.5 hr stirring, and are maximum as they are kept constant even after 2.5 hrs. KO_2 and Na_2O_2 were readily soluble.

Figure legends

Fig. 1. Metal superoxide (**a**) and peroxide/hydroperoxide (**b**) hydrolysis reaction mechanisms depicted by Lewis electron dot diagrams:

1a. Hydrolysis of metal superoxides: In a $Me^{+}O_2^{-}$ type superoxide (e.g., KO_2), one mole of H_2O dissociates the metal ion (reaction 2 in the text), freeing the superoxide radical anion ($O_2^{\bullet-}$), which due to its extra electron is electrophilically attacked at one end by the water H^+. Electrons are attracted to that end of the $O_2^{\bullet-}$ ion, and the oxygen atom (O) at its other end departs, leaving behind an atomic oxygen free radical ($O^{\bullet-}$). Then, the H^+ ion bonds to the $O^{\bullet-}$ radical ion that it has approached (and forms a hydoxyl radical, $HO^{\cdot}$), leaving the OH^- ion behind. From two moles of $Me^{+}O_2^{-}$, two OH^- ions are generated and combine with the two free Me^+ ions to generate two moles of MeOH, two $HO^{\cdot}$ radicals that combine with each other to generate one mole of H_2O_2, and two very reactive O atoms that combine in one mole of O_2.

2b. Hydrolysis of metal peroxides/hydroperoxides: In a type $Me_2^{+}O_2^{2-}$ peroxide (e.g., Na_2O_2), one mole of H_2O dissociates its two Me^+ ions (reaction 4 in text), freeing the peroxide anion (O_2^{2-}), which due to its two extra electrons is electrophilically attacked at each end by the water H^+ ion, and is split in two $O^{\bullet-}$ radical ions. Each of these is bonded by a H^+ ion to generate two $HO^{\cdot}$ radicals, which, in turn, combine to generate one mole of H_2O_2, leaving the two OH^- ions to combine with the two free Me^+ ions and generate two moles of MeOH. The hydrolysis mechanism of metal hydroperoxides (MeOOH; reaction 6 in the text) proceeds also via peroxide anion (O_2^{2-}) electrophilic attack.

Fig. 2. Evaluation of the reaction conditions for $O_2^{\bullet-}$ with the OR assay enzymes cyt. c_{ox} and SOD: (**a**) To test the effectiveness of cyt. c_{ox} in scavenging $O_2^{\bullet-}$, the ratio of cyt. $c_{ox}/O_2^{\bullet-}$ was varied, and the inhibition that cyt. c_{ox} caused on the formation of H_2O_2, the product of the dismutation of $O_2^{\bullet-}$ by H_2O, as measured by the *HRP-HVA assay*, was converted to % scavenged $O_2^{\bullet-}$. (**b**) The effect of

perchlorate (110 mM ClO_4^-, in 0.25 M K-phosphate buffer, pH 7.2) was tested (i) on the dismutation of 10 μM $O_2^{\bullet-}$ to H_2O_2 by SOD (45 U ml^{-1}) in the K-phosphate buffer, and (ii) on the $O_2^{\bullet-}$ % scavenging by 10x molar excess cyt. c_{ox} (100 μM). The resulting H_2O_2 concentrations (measured with the *HRP-HVA assay*) from treatments (i) and (ii) were 5 and 0 μM, respectively (based on the stoichiometry of reactions 1 and 9), as expected for a non-interfering effect of perchlorate on both $O_2^{\bullet-}$ dismutation by SOD and scavenging by cyt. c_{ox}. Error bars designate SE.

Fig 3. Stability of H_2O_2/CAT and their O_2 release reaction in the OR assay as a function of pH, the Martian soil constituents carbonate and perchlorate, and phosphate: Stability data of H_2O_2 at 0.5 mM are shown (they are same as at 0.1 mM), (**a**) in 0.25 M K-phosphate buffer (initially set at pH 7.2), containing 50 mM sodium carbonate (data, not shown, were the same in the absence of carbonate), and (**b**) in 50 mM sodium carbonate alone (here, tested pH's were set with NaOH). Perchlorate was tested on H_2O_2 stability and on CAT activity (50 U ml^{-1}) at pH 7.2 (in the K-phosphate-buffer), (**c**) in 0.5 mM H_2O_2 and 110 mM perchlorate ±CAT, and (**d**) in 0.1 mM H_2O_2 and 25 mM perchlorate ±CAT. Error bars designate SE.

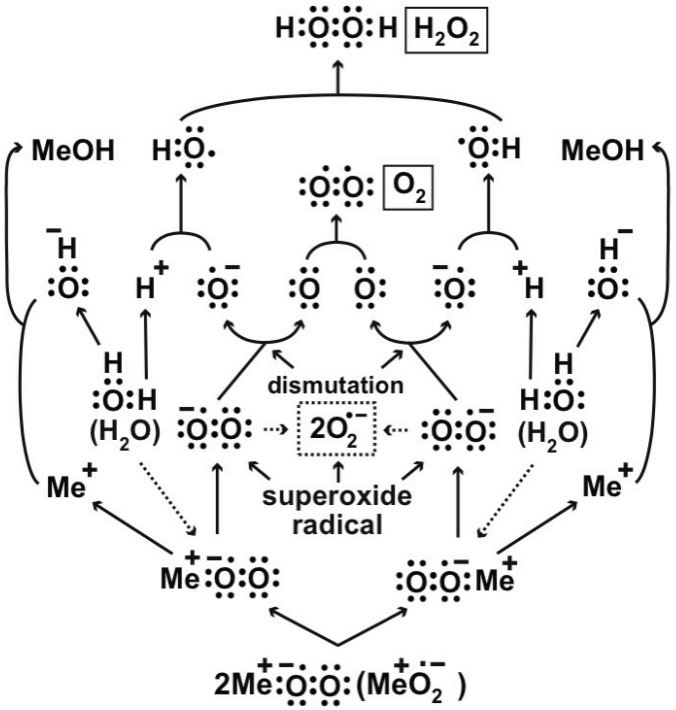
Fig. 4. Effect of *γ*-radiation on SOD, CAT and cyt. c_{ox}: (**a**) Effect of various doses of *γ*-ray on the specific activity of solid SOD and CAT, and on the specific reducibility of cyt. c_{ox}, all protein reagents dissolved in 100 mM K-phosphate buffer, pH 7.2. (**b**) Effect of various ACN concentrations (% v/v) on the same functionalities of the radiation unexposed proteins (dissolved in the same K-phosphate buffer). Error bars designate SE.

Fig. 5. Simulation of the OR assay on $O_2^{\bullet-}$ and H_2O_2: It is initiated (**a**) by the addition of 50 nmol $O_2^{\bullet-}$ in the absence and presence of 45 units SOD and the concentration of O_2 (from the boxed $O_2^{\bullet-}$-dismutation reaction) is recorded (reading A_{dism}, *treatment A* in text). In a subsequent step (**b**; reading $A_{dism/CAT}$, *treatment B*), addition of CAT causes additional O_2 release from the

decomposition of H_2O_2 (the second dismutation product of $O_2^{\bullet-}$), while an extra addition of 40 nmol H_2O_2 is also decomposed to O_2 by CAT (shown by the boxed reaction); *B* reading represents the concentration sum of the three consecutive releases of O_2 in step **b**. In a separate O_2-electrode recording (**c**; reading $B_{cyt.c}$, *treatment C*), the added 50 nmol $O_2^{\bullet-}$ are oxidized to equal nmol O_2 by cyt. c_{ox} already present in the O_2 electrode chamber. When the released O_2 concentration values A_{dism}, $A_{dism/CAT}$, and $B_{cyt.c}$, are inserted to the molar equations $O_2^{\bullet-} = 2(B_{cyt.c} - A_{dism})$ and $H_2O_2 = 2A_{dism/CAT} - (B_{cyt.c} + A_{dism})$ (see sub-section '*OR assay*' in '*Experimental*' section), the calculated experimental concentrations of $O_2^{\bullet-}$ and H_2O_2 (54 and 37 nmol, respectively) are statistically equal to the concentrations of $O_2^{\bullet-}$ and H_2O_2 (50 and 40 nmol, respectively), initially added in the O_2 electrode chamber.

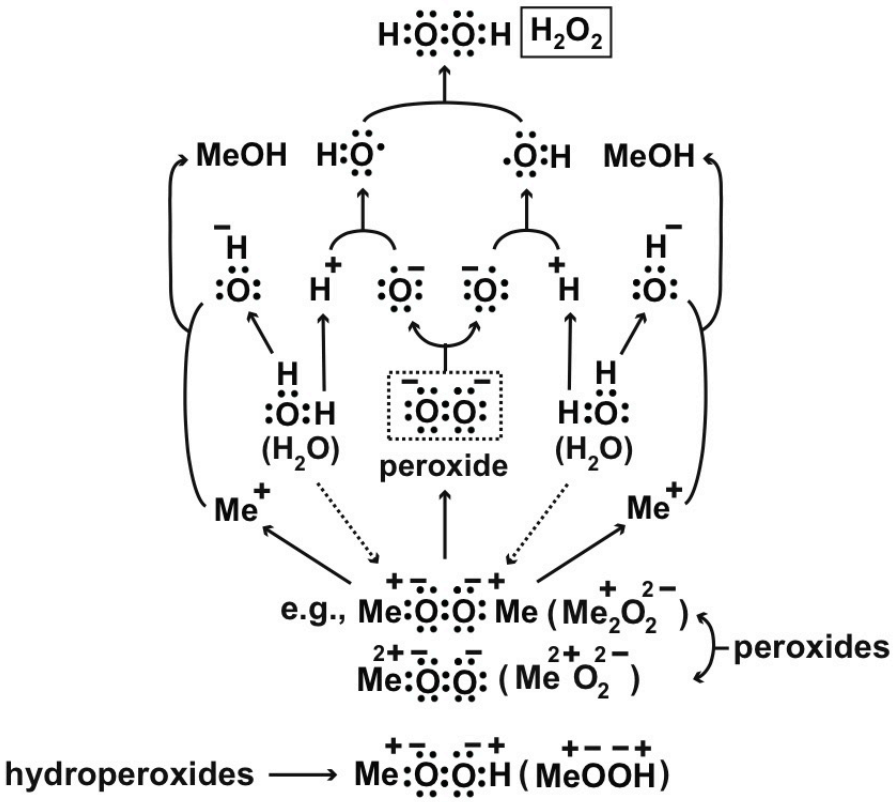
Fig. 6. Diagrammatic principle of the field OR assay: Soil sample is subjected to two separate O_2 measuring *procedures* (*A*, *B*), in each of which the sample is subjected (at constant mixing) to two different treatments (depicted as boxes within of which the specific reactions that take place are also drawn). In treatment A_{dism} (in *procedure A*), the soil is wetted (with K-phosphate-DTPA buffer, pH 7.2) and O_2 is released (recorded by the chamber O_2 sensor) by the SOD-catalyzed dismutation of $O_2^{\bullet-}$ (resulting from the hydrolysis of metal superoxides). Subsequently, in the same *procedure* a new treatment ($A_{dism/CAT}$) takes place, where to the already wetted soil CAT is added and additional O_2 is released from the CAT-decomposition of H_2O_2 coming from the hydrolysis of both metal superoxides and peroxides/hydroperoxides. In *procedure B*, with treatments $B_{cyt.c}$ or $B_{cyt.c/CAT}$ the soil is mixed (just wetted) with the same buffer supplemented with either cyt. c_{ox}, or is subsequently mixed with CAT, respectively. In the first treatment, O_2 is released from the reaction of $O_2^{\bullet-}$ with cyt. c_{ox}, while in the second treatment additional O_2 is released from the CAT-decomposition of H_2O_2 that came from the hydrolysis of metal peroxides/hydroperoxides. After each treatment, the moles (*x* and *y*, from metal superoxides and peroxides/hydroperoxides, respectively) of released O_2 (shown in squares; the doted ones depict zO_2 possibly coming from other sources) are recorded.

Figure 1a



Hydrolysis of metal superoxides proceeds via dismutation of the intermediate superoxide radical anion, $O_2^{\cdot -}$ ($:\ddot{\underset{\cdot\cdot}{O}}:\ddot{\underset{\cdot\cdot}{O}}:^{-}$)

Figure 1b



Hydrolysis of metal peroxides and hydroperoxides proceeds via intermediate peroxide anion, O_2^{2-} ($:\ddot{O}:\ddot{O}:$), nucleophilic splitting

Figure 2

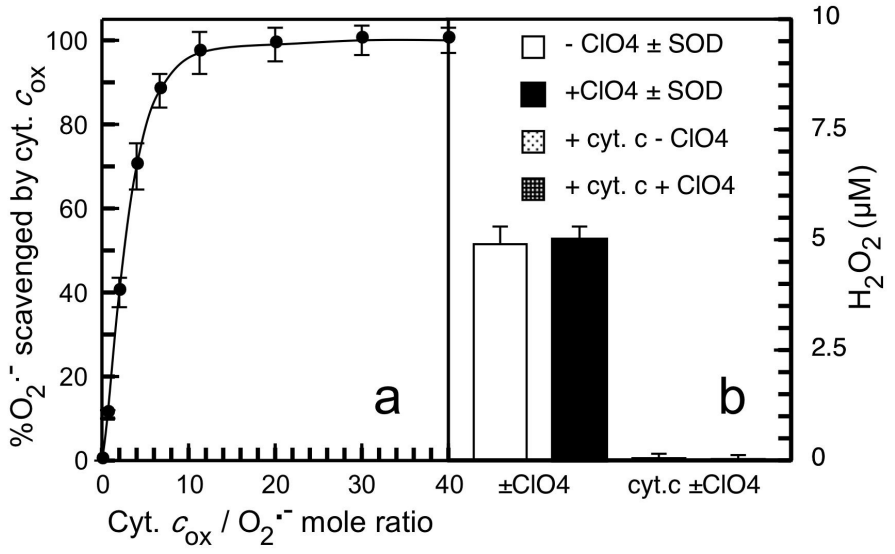


Figure 3

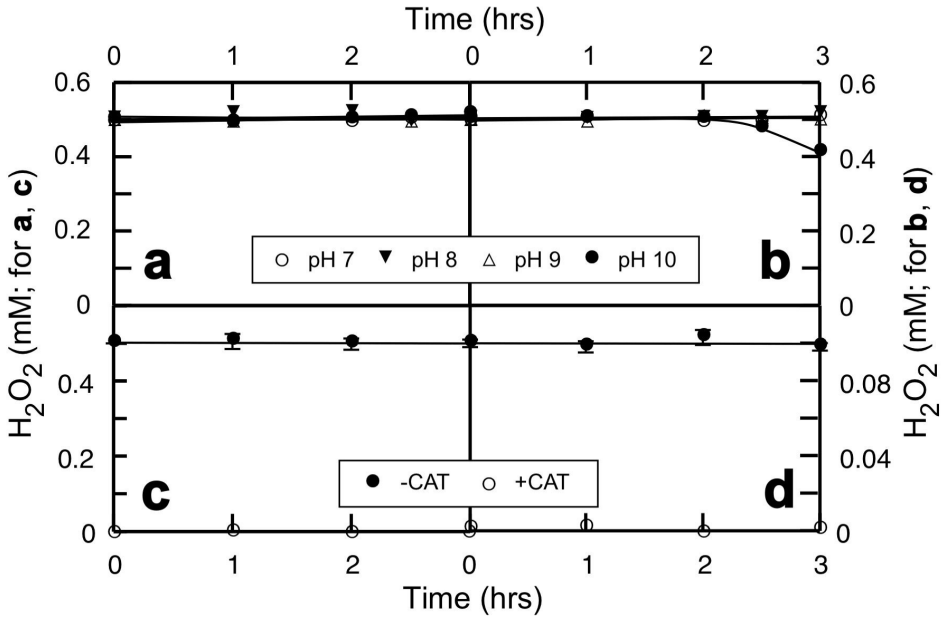


Figure 4

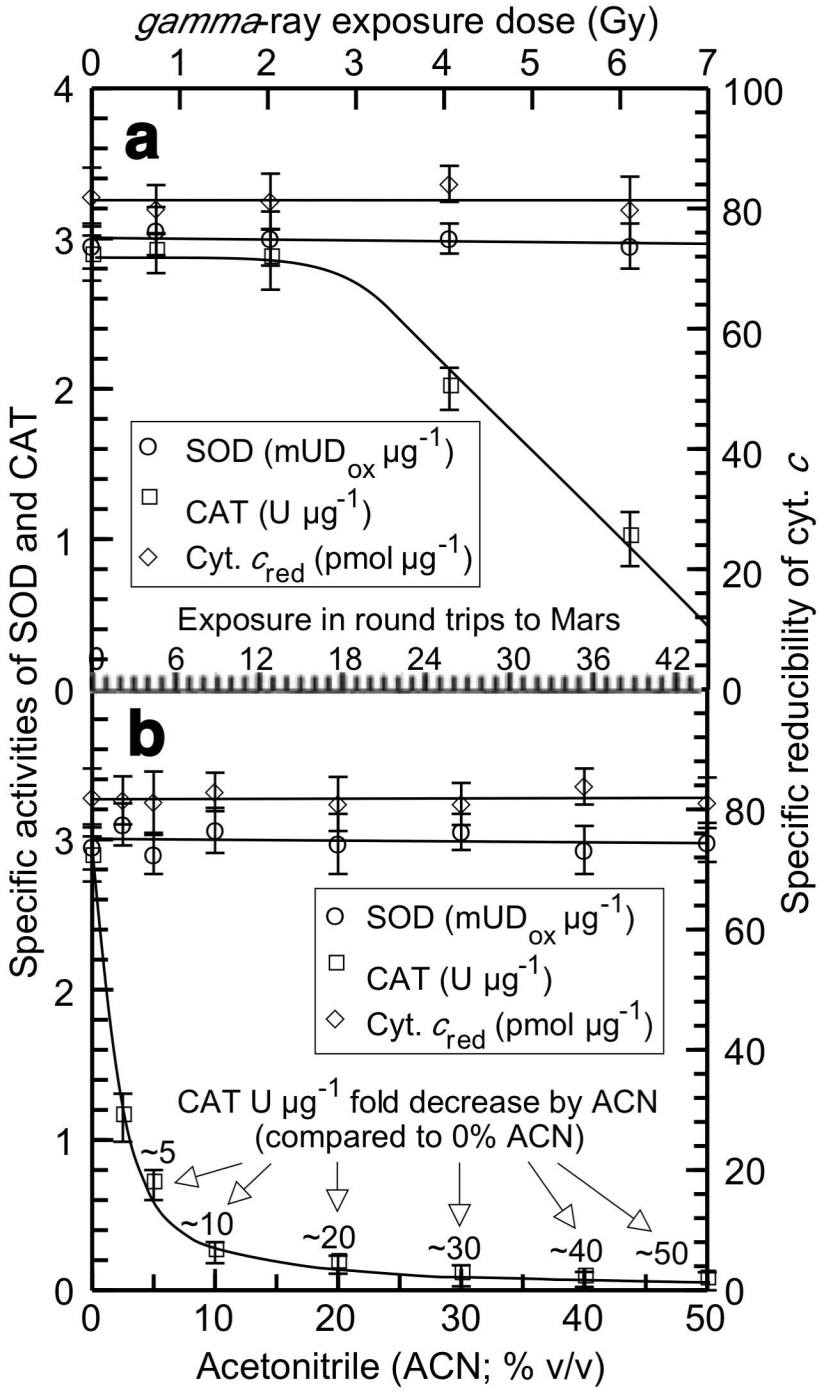

gamma-ray exposure dose (Gy)
0 1 2 3 4 5 6 7
a
SOD (mUD$_{ox}$ µg^{-1})
CAT (U µg^{-1})
Cyt. c_{red} (pmol µg^{-1})
Exposure in round trips to Mars
0 6 12 18 24 30 36 42
b
SOD (mUD$_{ox}$ µg^{-1})
CAT (U µg^{-1})
Cyt. c_{red} (pmol µg^{-1})
CAT U µg^{-1} fold decrease by ACN
(compared to 0% ACN)
~5
~10
~20
~30
~40
~50
Specific activities of SOD and CAT
Specific reducibility of cyt. *c*
0 1 2 3 4
0 20 40 60 80 100
0 10 20 30 40 50
Acetonitrile (ACN; % v/v)

Figure 5

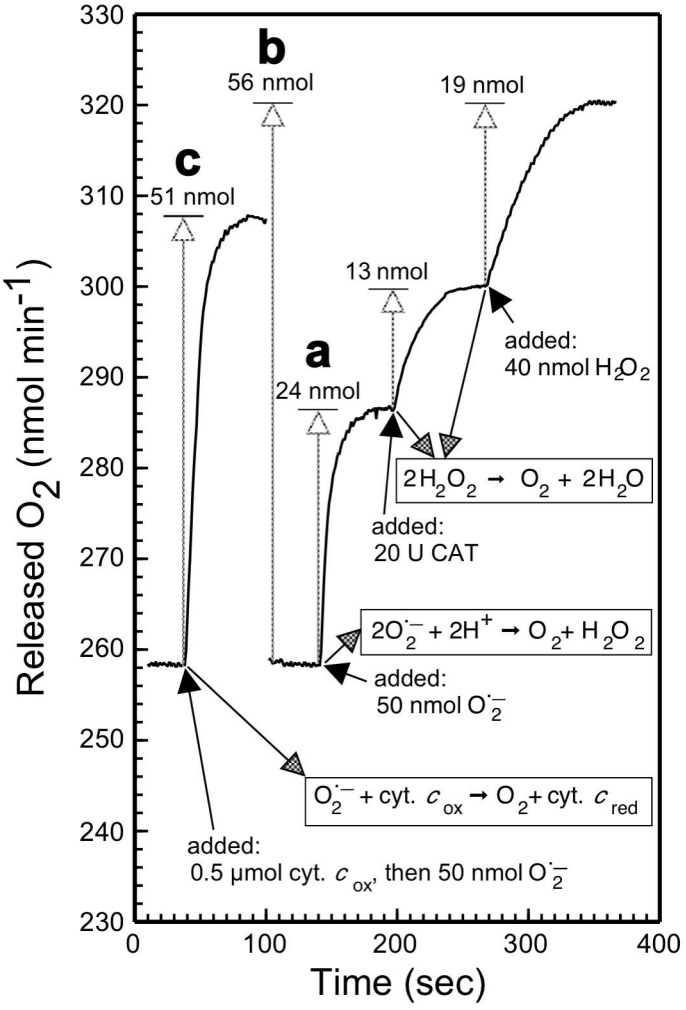
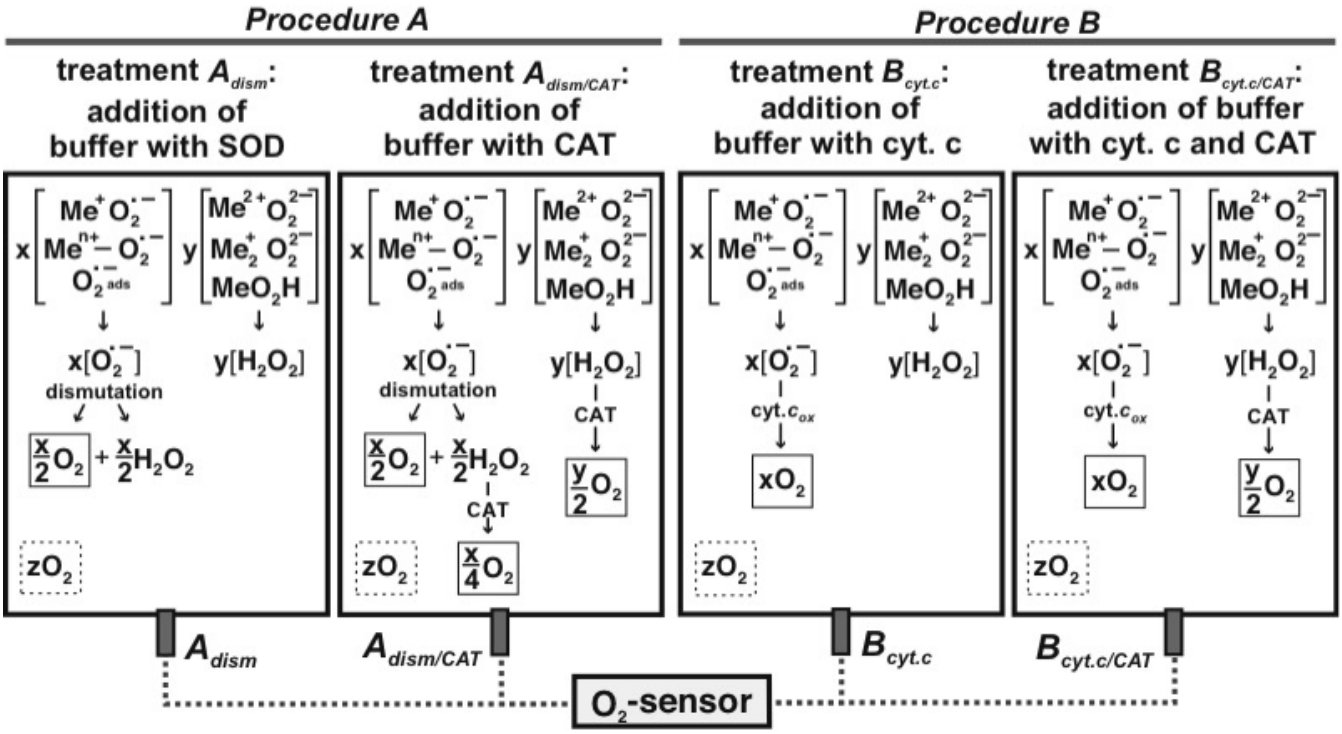

b
56 nmol
19 nmol
c
51 nmol
13 nmol
a
24 nmol
added:
40 nmol H2O2
2H2O2 → O2 + 2H2O
added:
20 U CAT
2O2·− + 2H+ → O2 + H2O2
added:
50 nmol O2·−
O2·− + cyt. c ox → O2 + cyt. c red
added:
0.5 µmol cyt. c ox, then 50 nmol O2·−
Released O2 (nmol min-1)
Time (sec)
330
320
310
300
290
280
270
260
250
240
230
0
100
200
300
400

Figure 6


Procedure A
Procedure B
treatment A_{dism}: addition of buffer with SOD
treatment $A_{dism/CAT}$: addition of buffer with CAT
treatment $B_{cyt.c}$: addition of buffer with cyt. c
treatment $B_{cyt.c/CAT}$: addition of buffer with cyt. c and CAT
dismutation
CAT
cyt.c_{ox}
A_{dism}
$A_{dism/CAT}$
$B_{cyt.c}$
$B_{cyt.c/CAT}$
O_2-sensor